University of Alberta
Printing Department

UNIVERSITY OF ALBERTA
LIBRARY

Regulations Regarding Theses and Dissertations

Typescript copies of theses and dissertations for Master's and Doctor's degrees deposited in the University of Alberta Library, as the official Copy of the Faculty of Graduate Studies, may be consulted in the Reference Reading Room only.

A second copy is on deposit in the Department under whose supervision the work was done. Some Departments are willing to loan their copy to libraries, through the inter-library loan service of the University of Alberta Library.

These theses and dissertations are to be used only with due regard to the rights of the author. Written permission of the author and of the Department must be obtained through the University of Alberta Library when extended passages are copied. When permission has been granted, acknowledgement must appear in the published work.

This thesis or dissertation has been used in accordance with the above regulations by the persons listed below. The borrowing library is obligated to secure the signature of each user.

Please sign below:

Date	Signature	Institution

Digitized by the Internet Archive
in 2019 with funding from
University of Alberta Libraries

https://archive.org/details/Diemer1965

Thesis
1965
#17

THE UNIVERSITY OF ALBERTA

STUDIES OF THE PRIMARY STRUCTURE OF 18S + 28S RIBONUCLEATES FROM WHEAT GERM

A THESIS
SUBMITTED TO THE FACULTY OF GRADUATE STUDIES
IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR
THE DEGREE OF MASTER OF SCIENCE

DEPARTMENT OF BIOCHEMISTRY

by

Jans Diemer

EDMONTON, ALBERTA

APRIL, 1965

UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "STUDIES OF THE PRIMARY STRUCTURE OF 18S + 28S RIBONUCLEATES FROM WHEAT GERM", submitted by Jans Diemer in partial fulfilment of the requirements for the degree of Master of Science.

Date April 2/65

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "STUDIES OF THE PRIMARY STRUCTURE OF 18S + 28S RIBONUCLEATES FROM WHEAT GERM", submitted by Jane [illegible] in partial fulfilment of the requirements for the degree of Master of Science.

....
Supervisor

....

....

....

....

Date ....

ABSTRACT

A technique for preparing large quantities of 18S + 28S ribonucleates from wheat germ has been developed. End group and sedimentation analyses were used to characterize the ribonucleates with respect to features of primary structure, heterogeneity and stability.

Hydrolytic degradation by alkali has been employed as a means of characterizing the native chain ends of the ribonucleates, and hydrolytic degradation by phosphodiesterase has been employed to characterize the nascent chain ends which result from scission of the ribonucleate chains.

ACKNOWLEDGEMENTS

The guidance and encouragement of Dr. B.G. Lane, who supervised these studies, is gratefully acknowledged. The author is also deeply grateful to Dr. Lane for contributing the section of the thesis dealing with the alkali hydrolysis of ribonucleates. The author wishes to express deep appreciation to Dr. C.M. Kay for the sedimentation analyses of the ribonucleates.

The generous cooperation of Mr. B.D. McLennan in many aspects of the work, and the assistance of Mr. C.A. Blashko in the desalting studies are gratefully acknowledged.

The planimetric analysis of the schlieren patterns by Miss G.M. Gullekson is sincerely appreciated. The author is grateful to Mr. J. Durgo for performing the ultracentrifuge runs.

The author wishes to acknowledge a pleasant association with members of the staff in the Department of Biochemistry.

Special thanks are due to Mrs. A. Bloom who typed the thesis.

TABLE OF CONTENTS

Page

Abstract iii

Acknowledgements iv

List of Tables vii

List of Illustrations ix

List of Abbreviations x

INTRODUCTION

(i) The structural chemistry of ribonucleates 1

(ii) The background of end group work on the cellular ribonucleates........................ 5

I. PREPARATIVE PROCEDURES:

(i) Preparation of 18S + 28S ribonucleates from wheat germ 15

(ii) Preparation of phosphodiesterase from Russell Viper Venom 16

(iii) Preparation of pNp compounds 18

(iv) Preparation of pN>p compounds 20

II. ANALYTICAL PROCEDURES:

(i) Assay of snake venom phosphodiesterase activity 22

(ii) Anion-exchange fractionation of alkali and phosphodiesterase hydrolyzates on DEAE-cellulose 24

(iii) Desalting of nucleate derivatives in the effluents from DEAE-cellulose columns 25

(iv) Separation of N, pNp and pN>p compounds by paper chromatography 27

III. ANALYSIS OF THE NATIVE END GROUPS OF 18S + 28S RIBONUCLEATES FROM WHEAT GERM;

(i) The rational and experimental basis of the analysis 29

(ii) The significance of the results for concepts of the chemical structure of 18S + 28S ribonucleates 32

Page

IV. ANALYSIS OF THE END GROUPS WHICH RESULT FROM PHOSPHODIESTER CLEAVAGE OF RIBONUCLEATE CHAINS:
- (i) Hydrolysis of ribonucleates by snake venom phosphodiesterase 34
  - (a) The rational and experimental basis for the method 34
  - (b) The effects of nuclease and phosphomonoesterase contamination of snake venom phosphodiesterase preparations 36
  - (c) An unusual side reaction with amino alcohol buffers 36
  - (d) The stability of pNp and pN>p compounds in the phosphodiesterase hydrolyzates 37
- (ii) End group measurements 40
  - (a) An extensively degraded ribonucleate specimen of commercial origin 40
  - (b) A partially degraded specimen of _E_. _coli_ high molecular weight ribonucleates 40
  - (c) Undegraded 18S + 28S ribonucleates from wheat germ 41
  - (d) Undegraded s-ribonucleates from wheat germ 44

V. THE CLEAVAGE OF 18S + 28S RIBONUCLEATES UNDER A VARIETY OF COMMONLY EMPLOYED CONDITIONS:
- (i) The effect of incubation at pH 9 and 37° 46
- (ii) The labilizing effect of magnesium counterion 47
- (iii) The effect of heating at 100° and neutral pH 48
- (iv) The hydrolysis rate at different pH values and temperatures 48

VI. THE STORAGE OF 18S + 28S RIBONUCLEATES IN THE POWDER STATE 50

VII. SUMMARY 52

BIBLIOGRAPHY 55

Appendix 58

LIST OF TABLES

Table Page*

I. The purity and activity of snake venom phosphodiesterase at various stages in the purification procedure.......... 18

II. Eluents used to fractionate alkali hydrolyzates of the 18S + 28S ribonucleates on DEAE-cellulose...... 25

III. Eluents used to fractionate phosphodiesterase hydrolyzates of the 18S + 28S ribonucleates on DEAE-cellulose.......... 25

IV. The desalting of effluents from DEAE-cellulose columns by adsorption to and elution from charcoal....... 26

V. The fractionation on DEAE-cellulose of a 90 hour alkali hydrolyzate of 18S + 28S ribonucleates from wheat germ.......... 29

VI. The end groups determined after alkali hydrolysis of the 18S + 28S ribonucleates from wheat germ........ 31

VII. The nucleosides and nucleoside diphosphates determined after alkali hydrolysis of degraded ribonucleate preparations.......... 31

VIII. Results of column fractionation on DEAE-cellulose of a typical phosphodiesterase hydrolyzate........... 35

IX. Results of end group analyses performed with snake venom phosphodiesterase preparations contaminated by nuclease or phosphomonoesterase activities......... 36

X. The yield of nucleosides and unusual phosphodiester derivatives formed during end group analyses in which amino alcohol buffers were employed............ 37

XI. Results of a typical experiment to determine the stability of pNp compounds in phosphodiesterase hydrolyzates.......... 37

XII. The effect of substrate concentration, product concentration and phosphodiesterase inhibitors on the hydrolysis of pNp compounds in the presence of phosphodiesterase.......... 37

XIII. The spontaneous hydrolysis of pN>p compounds at pH 9.2 and 37^{o}.......... 38

XIV. The end groups determined by phosphodiesterase hydrolysis of a badly degraded specimen of yeast ribonucleates from a commercial source................ 40

XV. The distribution of bases in the end groups determined by snake venom phosphodiesterase hydrolysis of fragmented 18S + 28S ribonucleates from *E. coli*.... 41

Page*

XVI. The end groups determined by snake venom phosphodiesterase hydrolysis of the 18S + 28S ribonucleates from wheat germ.................................... 41

XVII. The end groups produced by spontaneous hydrolysis of ribonucleates during end group analyses with phosphodiesterase calculated from the time-course of hydrolysis and the observed mean rate of spontaneous hydrolysis.................................... 42

XVIII. The effect of incomplete hydrolysis by phosphodiesterase on the end group measurements............. 42

XIX. Nucleoside end groups determined by snake venom phosphodiesterase hydrolysis of s-ribonucleates from wheat germ.................................... 44

XX. The end groups produced by spontaneous hydrolysis of 18S + 28S ribonucleates at pH 9.2 and 37°....... 46

XXI. The end groups produced by spontaneous hydrolysis of 18S + 28S ribonucleates at different pH values and temperatures.................................. 48

XXII. Rates of hydrolysis of 18S + 28S ribonucleates at various pH values and temperatures, based on measurements of nucleoside end groups after phosphodiesterase hydrolysis.................................. 48

XXIII. End group and sedimentation analyses of the 18S + 28S ribonucleates stored in the powder state....... 50

XXIV. Sedimentation analyses of 18S + 28S ribonucleates after storage in the powder state at -20°.......... 50

---

* The tables precede the pages indicated.

LIST OF ILLUSTRATIONS

Figure Page*

1. Ultraviolet photographs of two-dimensional paper chromatograms which depict the separation of nucleosides and diphosphonucleosides derived from 90 hour alkali hydrolyzates of the 18S + 28S ribonucleates from wheat germ........................ 29

2. Ultraviolet photograph of a one-dimensional paper chromatogram depicting the separation of the nucleosides obtained from a 43 hour alkali hydrolyzate of the 18S + 28S ribonucleates from wheat germ... 30

3. Paper chromatographic resolution and spectral analysis of the nucleosides and diphosphonucleosides derived from phosphodiesterase hydrolyzates of the 18S + 28S ribonucleates from wheat germ............ 35

4. Ultraviolet photographs of paper chromatograms showing the hydrolysis of pN>p compounds after various periods of time at pH 9.2 and 37°.......... 38

5. Ultraviolet photographs illustrating the one-dimensional paper chromatographic resolution of pN>p and pNp compounds........................... 48

6. Schlieren photographs from sedimentation studies.... 50

* Illustrations precede the pages indicated.

ABBREVIATIONS

N - nucleoside (e.g. A = adenosine)

pN - nucleoside 5'-monophosphate

Np - nucleoside 2'(3')-monophosphate

pNp - 3',5'-diphosphonucleoside

pN>p - 2'-3'cyclic,5'-diphosphonucleoside

Nxp - 2'-O-methyl-nucleoside 3'-phosphate

RNA - ribonucleates

s-ribonucleates - soluble ribonucleates

DEAE-cellulose - diethylaminoethyl-cellulose

s.u. - spectrophotometric units (=O.D. x volume)

TRIS - Tris(hydroxymethyl)-aminomethane

DIOL - 2-amino-2-methyl-1,3-propanediol

TCA - trichloroacetic acid

AC - ammonium chloride

AF - ammonium formate

DF - DIOL-formate

HC - hydrochloric acid

PP - potassium phosphate

SC - sodium chloride

SH - sodium hydroxide

TF - TRIS-formate

INTRODUCTION

(i) The structural chemistry of ribonucleates.

The progress made toward the chemical characterization of ribonucleates during the fifty years which followed their discovery by Kossel, in 1879, can be attributed to the feasibility of their isolation in large quantities from yeast and wheat germ (see Allen, 1962). It is no less true today that progress in the structural chemistry of the ribonucleates relies on their availability in large quantity from these two sources (Holley, 1963; Glitz and Dekker, 1963). The early chemical studies demanded large amounts of material in order to identify the hitherto uncharacterized nucleotide constituents of the ribonucleates by the methods of classical organic chemistry. These early studies, and even the more recent studies which established the nature of the 3'-5' internucleoside phosphodiester linkage, did not require that the ribonucleates be isolated in an undegraded form. Indeed, the principal features of the covalent structure of ribonucleate chains had been established (Brown and Todd, 1952a, 1952b, 1954; Markham and Smith, 1952a, 1952b; Cohn and Volkin, 1952) before the first isolation of highly polymerized, undegraded cellular ribonucleates was achieved by Colter and Brown in 1956.

Further progress toward elucidating the chemical structure of the highly polymerized ribonucleates requires the development of methods for their large-scale isolation in an undegraded state and the establishment of conditions which permit their storage without degradation over reasonable

periods of time. Wheat germ has been selected as a source material for highly polymerized ribonucleates in this study because it is readily available in unlimited quantity, and is a naturally dry organism which can be stored for periods of up to two years without noticeable deterioration of the ribonucleates which comprise 1% of the dry weight of the embryo. The highly polymerized 18S + 28S ribonucleates from wheat germ are quite labile after isolation from the embryo and conditions for storage in the powder state without degradation have been established.

With the covalent structure and polymeric nature of the ribonucleates well-established, there are four principal features of primary structure which remain to be investigated: (i) the number of recognizable size-classes of ribonucleates, (ii) the nature of the end groups of the chains in each size-class, (iii) the number of different chains in each size-class, (iv) the sequence of nucleotides in each of the different chains in each size-class.

Only the first of these aspects of the structural chemistry of ribonucleates seems to have been adequately resolved for the bulk of the cellular ribonucleates. The low molecular weight s-ribonucleates account for about 20% of the total cellular ribonucleates and have a mean chain length of approximately 80 nucleotide residues. The high molecular weight 18S + 28S ribonucleates account for about 80% of the total cellular ribonucleates and have a mean chain length of about 1300 nucleotide residues.

Because of their well-defined participation in

protein synthesis and their low molecular weight which makes them amenable to fractionation and sequence studies, the s-ribonucleates have been studied much more extensively than the 18S + 28S ribonucleates with respect to the remaining three aspects of structural chemistry outlined earlier. The nature of the end groups was established soon after the s-ribonucleates were discovered (Dunn, 1959). There are believed to be at least 20 different chains, many of which have been separated from the bulk of the s-ribonucleates (Goldstein *et al.*, 1964). The complete nucleotide sequences of individual s-ribonucleate molecules are being investigated in several laboratories at the present time (Zubay, 1962; Cantoni *et al.*, 1963; Holley *et al.*, 1964).

In contrast with the s-ribonucleates, there has been no evidence bearing on the end groups, number of chains, or sequences within chains in the case of the high molecular weight 18S + 28S ribonucleates, and the studies of this thesis constitute a first step toward resolving some of these considerations. There are manifold reasons for this neglect of the 18S + 28S ribonucleates, not the least of which is the inability to ascribe a functional significance to them in cellular metabolism. Perhaps of equal importance however have been the technical difficulties of isolating large amounts of undegraded material which does not disintegrate when stored or subjected to the mildest procedures suitable for chemical investigations.

The requirement of large quantities of undegraded material for controlled end group studies is intrinsic to the

study of any polymers such as the 18S + 28S ribonucleates which are comprised of more than one thousand monomer units and in which the end groups account for less than 0.1% of the total monomer constituents. In order to distinguish native end groups from those produced by degradation of the polymers it is important to isolate the polymers in an undegraded state, and, if possible, to distinguish qualitatively as well as quantitatively between the native end groups and those which result from degradation. The studies of this thesis have been primarily concerned with developing methods for the investigation of end groups of the 18S + 28S ribonucleates and it has been found that the native end groups can be measured and distinguished from those arising from polymer degradation.

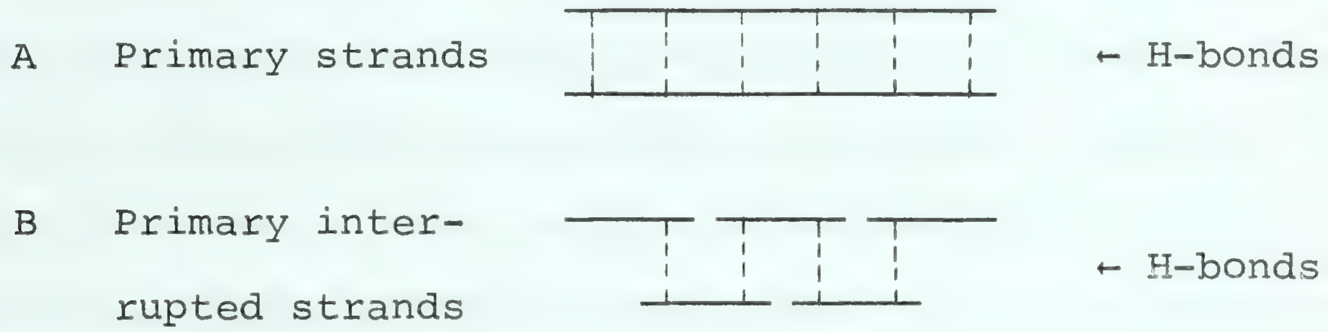
The results of analyses for the native end groups of the 18S + 28S ribonucleates have shown that they consist of at least four different chains having a mean chain length of 1300 nucleotide residues. The studies of the end groups produced by the scission of phosphodiester bonds in the 18S + 28S ribonucleates have focused attention on a serious impediment to fractionation and sequence studies on these ribonucleates: the intrinsic instability of 3'-5' phosphodiester linkages in ribonucleate chains. It has become clear that the tendency of the 18S + 28S ribonucleates to disintegrate under mild conditions is an intrinsic property resulting from the instability of the 3'-5' phosphodiester linkages which are adjacent to 2'-OH functions and readily form 2'-3' cyclic phosphates with concommitant cleavage of

the 3'-5' phosphodiester bonds. The instability of phosphodiester linkages is particularly apparent with the 18S + 28S ribonucleates because the cleavage of 0.1% of the phosphodiester linkages causes an average of more than one break in every chain whereas the cleavage of 0.1% of the phosphodiester linkages in s-ribonucleate preparations causes a break in fewer than one in every ten chains.

(ii) The background of end group work on the cellular ribonucleates.

The importance of end group measurements in studies which relate to the primary structure of polymers can be appreciated from a consideration of the conflicting views of deoxyribonucleate structure which were held as recently as 1954. Physicochemical measurements had shown that the cellular deoxyribonucleates had a mean molecular weight of about 5×10^6, and this value, together with the Watson-Crick proposal of a double-stranded helix, suggested that the primary structures were two single strands each of which contained about 7500 nucleotide residues. This view of deoxyribonucleate structure conflicted with titration data which indicated that one of every fifty phosphate groups was a phosphomonoester group, and in order to resolve this conflict, Dekker and Schachman (1954) proposed an "interrupted strand" modification of the Watson-Crick model in which the primary structures were thought to be short chains about 50 nucleotides long having terminal phosphomonoester groups. The short chains were conceived to be

held together by secondary hydrogen bonds between the interrupted strands of a double-helical structure of the Watson-Crick type. The conflicting views of structure are schematically represented by the Watson-Crick model (A) and the Dekker-Schachman model (B) shown below for a segment of the deoxyribonucleate double helix.



It is clear that structure A has only two end groups per 7500 nucleotides whereas structure B has two end groups per 50 nucleotides and consequently end group measurements could have provided the decisive evidence required to validate one or other of the proposed structures. Methods for estimating the end groups of deoxyribonucleates have not been developed even now, and the resolution of the conflict in favor of structure A was only made possible by subsequent investigations which showed that the titration data were artifactual (Doty and Rice, 1955). A modified version of the Dekker-Schachman model may yet be invoked to explain the occurrence of deoxyribonucleate "subunits" (Welsh, 1962) but in this case the primary structures would be about 750 rather than 50 nucleotides long. The importance of end group measurements to biochemical and genetic studies of

deoxyribonucleates is increasingly evident from the central role of end groups in hypothetical proposals concerning chain initiation of deoxyribonucleate synthesis, cross-over phenomena and the repair of chain breaks in deoxyribonucleates (Taylor, 1963; Richardson et al., 1964).

The establishment of the 3'-5' phosphodiester internucleoside linkage and the polymeric nature of the nucleates permits numerous formal structures to be written for the primary structural units of the polymers without regard to sequential arrangement of monomers, and these different structures are distinguished solely by the nature of the end groups. This thesis deals exclusively with ribonucleates but the principles to be discussed here would be, for the most part, equally applicable to a consideration of deoxyribonucleates.

Since the ribonucleates can be largely converted to 5'-nucleotides by snake venom phosphodiesterase, they might be thought of as being polymers of 5'-nucleotides (ie. $(pN)_n$) as shown in structure I.

I pNpN-------pNpN-------pNpN

Alternatively, since the ribonucleates can be largely converted to 3'-nucleotides by spleen phosphodiesterase, they might be thought of as being polymers of 3'-nucleotides (ie. $(Np)_n$) as shown in structure II.

II NpNp-------NpNp-------NpNp

It can be seen that each of the structures, I and

II, has a terminal group linked to the remainder of the chain through its 3'-position ("left-hand" termini as written above) and another end group linked to the rest of the chain through its 5'-position ("right-hand" termini), and these will be designated as the 3'-linked end group and the 5'-linked end group, respectively.

It is evident that hydrolysis of structure I by snake venom phosphodiesterase would yield only 5'-nucleotides but hydrolysis of structure II by the same enzyme would yield 5'-nucleotides (pN) from internal residues, a nucleoside (N) from the 3'-linked end group and a diphosphonucleoside (pNp) from the 5'-linked end group. Consequently this type of hydrolysis could be used to assess the end groups of chains having a type II structure but not to assess the end groups of chains having a type I structure.

Conversely, hydrolysis of structure II by spleen phosphodiesterase would yield only 3'-nucleotides but hydrolysis of structure I by the same enzyme would yield 3'-nucleotides (Np) from internal residues, a nucleoside (N) from the 5'-linked end group and a diphosphonucleoside (pNp) from the 3'-linked end group. Therefore this enzyme could be used to assess the end groups of type I, but not to assess the end groups of type II structures.

In practice, snake venom phosphodiesterase has been adapted for the analysis of the end groups of type II structures but the spleen enzyme is not used for the analysis of the end groups of type I structures because alkali hydrolysis is simpler, in practice, and yields the same results

except that there is an isomerization to give roughly equal amounts of 2'- and 3'-nucleotides and 2',5'- and 3',5'-diphosphonucleosides.

These principles of end group analysis were first enunciated by Markham and Smith (1952) and Crestfield and Allen (1956), and it was also noted that the phosphomonoester group of structure II might be cyclized to give a third structure shown below as III.

III NpNp-------NpNp-------NpN>p

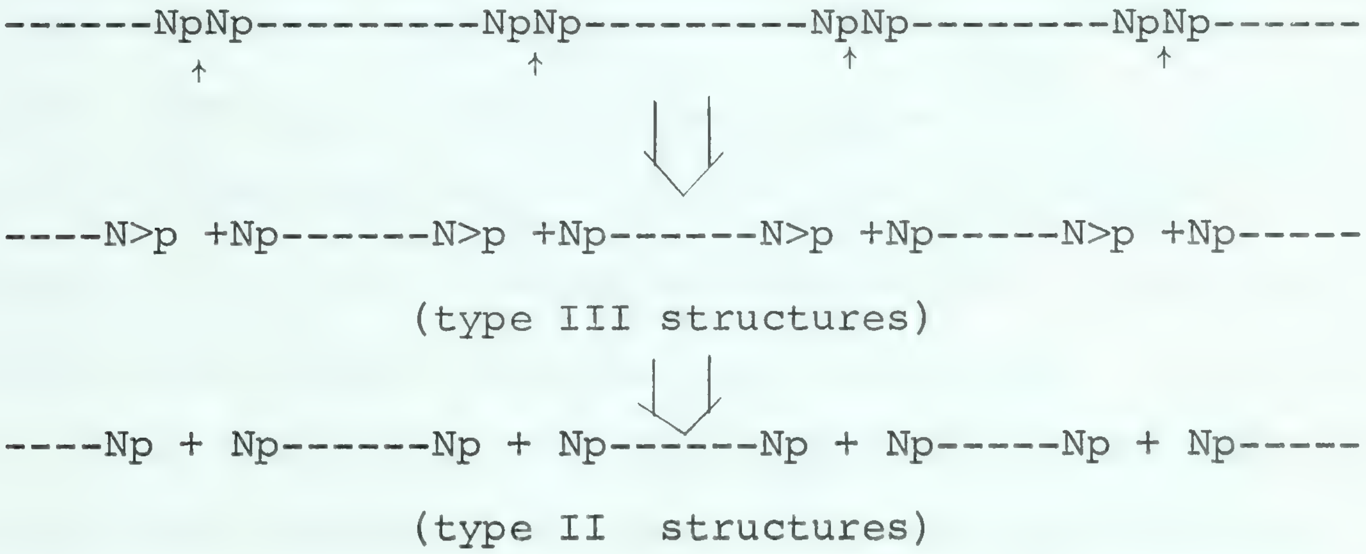
Hydrolysis of a type III structure by snake venom phosphodiesterase would yield 5'-nucleotides from internal residues, a nucleoside from the 3'-linked end group and a diphosphonucleoside having a cyclic 2',3' phosphate (pN>p) from the 5'-linked end group (Fraenkel-Conrat and Singer, 1962).

Other structures can be visualized which are hybrid forms of I, II and III with respect to end groups and are shown below as IV, V and VI.

IV pNpN-------pNpN-------pNpNp
V pNpN-------pNpN-------pNpN>p
VI NpNp-------NpNp-------NpN

In practice structures IV, V and VI are not important since the quantities of the 3'- and 5'-linked end groups detected by phosphodiesterase hydrolysis are equimolar and unrelated to the equimolar amounts of the 3'- and 5'-linked end groups detected by alkali hydrolysis.

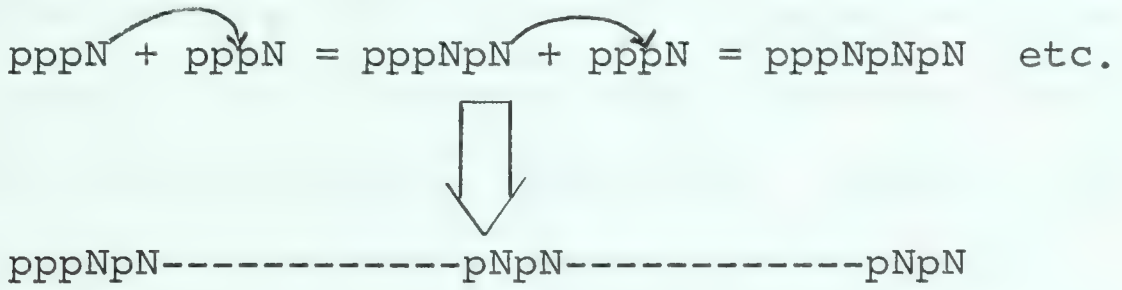
The early end group work by Markham and Smith (1952) and Crestfield and Allen (1956) is largely of historical interest since the yeast ribonucleates examined were badly degraded and had chain lengths of about 20-70 nucleotides. Nevertheless, it became apparent from this work that the structures formed by chemical and enzymic degradation were principally types II and III regardless of the nature of the native end groups of the ribonucleates. The reason for this is clear from a consideration of the mechanism by which chemical agents and nucleases induce cleavage of the 3'-5' phosphodiester linkages of ribonucleates.



There are no chemical agents and relatively few enzymes which can cleave 3'-5' phosphodiester linkages in the manner of snake venom phosphodiesterase to produce type I structures.

Studies of s-ribonucleates from animal, plant and microbial sources have shown that they have a type I structure with guanine as the principal base of the 3'-linked end groups and adenine as the principal base of the 5'-linked end groups. The type I structure is sim-

ilar to the structure which might be expected for the polymers synthesized by the deoxyribonucleate-directed, enzymically catalyzed pyrophosphorolytic condensation of ribonucleoside triphosphates (Kornberg, 1957) except that one might anticipate a triphosphate group rather than a phosphomonoester group at the 3'-linked end group. This is briefly illustrated below.



If ribonucleoside triphosphates are the immediate precursors of both terminal and non-terminal groups of the s-ribonucleates then there must be an as yet undefined release of pyrophosphate from the 3'-linked end group which is perhaps associated with chain initiation or termination.

Since actinomycin inhibits <u>all</u> synthesis of cellular ribonucleates through interaction with deoxyribonucleate (Reich *et al*., 1962), it seemed not unlikely that all ribonucleates were synthesized by the same mechanism and that the 18S + 28S ribonucleates might also be expected to be type I structures. This view has been confirmed by showing that the 18S + 28S ribonucleates from wheat germ are type I structures having about 1300 nucleotide residues, on the average. The number average molecular weight is somewhat lower than the weight average molecular weight by physicochemical techniques; the mean chain length is 1300 nucleo-

tides by end group measurements and about 2000 nucleotides on the basis of physicochemical data. The agreement is reasonable considering the uncertainties which attend molecular weight determinations by physicochemical methods since the hydrodynamic properties of high molecular weight ribonucleates are not yet well understood.

This view of the primary structure of the s-ribonucleates and the 18S + 28S ribonucleates as linear polymers having a type I structure with mean chain lengths of 80 and 1300 nucleotides, respectively, implies that there are no type II structures present in the polymers. This presumption could not be accepted without reservation however since the possibility of an "interrupted" strand structure for the ribonucleates was implicit from many investigations on the ribonucleates. The interrupted strands could be visualized in the following way:

```
           NpNp-----NpNp        NpNp------NpNp
           | |      | |         | |       | |
pNpN-------NpNp     NpNp--------NpNp      NpNp-----pNpN
```

The secondary hydrogen-bonds holding the interrupted strand together as an integral structure would be visualized as intra- rather than inter-strand (as with deoxyribonucleates) and such a single strand has frequently been described as an intra-strand hydrogen bonded structure with "hidden" breaks since the breaks would not be evident until the structure disintegrated (Spirin, 1960). The greater part of the work in this thesis was devoted to developing methods for the analysis of end groups of type II structures in order to

test the foregoing interrupted strand concept of ribonucleate structure.

It was noted earlier that the instability of 18S + 28S ribonucleate preparations has been a serious impediment to progress in the structural chemistry of these polymers since it has not been clear if the observed disintegration reflected the disruption of secondary forces with dissociation of short chains from an aggregate structure such as an interrupted strand, or, if there were a cleavage of the 3'-5' phosphodiester linkages of a single long chain whose integral structure did not rely on secondary forces. There were numerous reports that the 18S + 28S ribonucleates disintegrated to give small 4-8S components, often called "subunits", when subjected to a number of gentle treatments which were not believed to cause cleavage of phosphodiester linkages. The formation of "subunits" was shown in the case of ribosomal ribonucleates, from liver (Takanami, 1958; Hall and Doty, 1959), from Ehrlich ascites cells (Brown et al., 1960), from yeast cells (Otaka et al., 1961; Chao, 1961) and from E. coli (Aronson and McCarthy, 1961). It was clear that the "subunits" were not type I structures since end group analysis of 8S preparations showed a mean chain length of about 1300 nucleotides whereas the expected length for 8S chains was 300 nucleotides or less (Lane and Allen, 1961). When techniques for the analysis of the chain ends of type II and III structures had been developed, it was established that the 8S "subunits" were mainly type II and III structures (Lane et al., 1963).

The 8S "subunits" could have been present in the original 18S + 28S ribonucleates, as segments of an interrupted strand held together by secondary forces, or they might have been absent from the 18S + 28S ribonucleates and produced by cleavage of phosphodiester bonds. Physicochemical methods could offer only indirect evidence to distinguish between dissociation and cleavage whereas end group measurements have provided unequivocal evidence that "subunits" result from fragmentation of 3'-5' phosphodiester linkages since the type II structures are absent prior to "subunit" formation and are present in the expected amounts after "subunit" formation.

By a similar but less extensive study it has been shown that s-ribonucleates are not interrupted strands of type II structures held together by secondary forces as had been implied by titration data which showed that one of every seven phosphate groups was singly esterified (Davis, 1962). It has been concluded that these titration data, as in the earlier studies of deoxyribonucleates, are probably artifactual.

The work of this thesis also deals with a number of unusual side-reactions encountered in analyses for the end groups of type II structures as well as measurements of the rate of 3'-5' phosphodiester cleavage of ribonucleates under a variety of conditions commonly employed in studies of ribonucleates.

PART I - PREPARATIVE PROCEDURES

(i) Preparation of 18S + 28S ribonucleates from wheat germ.

Ninety grams of roller-milled wheat germ were suspended in 1200 ml of an emulsion made by mixing equal volumes of 0.05 M phosphate buffer, pH 6.8 and water-saturated phenol. The suspension was shaken vigorously for 15 min at room temperature and then centrifuged at 1500 g for 20 min at 0°. The emulsion separated into an upper aqueous phase containing ribonucleates and polysaccharides, a lower phenol phase containing protein, deoxyribonucleates and cellular debris, and an interphase of solid material. The upper aqueous phase was removed by suction, care being taken to avoid the inclusion of any solid material from the interphase. The aqueous solution was extracted five times with an equal volume of ether to remove phenol. The traces of ether remaining after these washings were removed by aeration of the solution at 0°. The solution was then made molar with respect to sodium chloride by addition of solid salt, and placed at 0° for 18 hours. The soluble ribonucleates and polysaccharides remained in solution and the high molecular weight ribonucleates precipitated. The precipitate was collected by centrifugation at 0°, and the resulting gel-like pellet was washed three times with 67% ethanol, three times with 95% ethanol and three times with ether. These washings removed salt, water and ethanol, respectively. The final ether wash was followed by a 30-minute air-drying to constant weight at room temper-

ature. The resulting powder was dissolved in distilled water at a concentration of 5 mg per ml and the ribonucleates were precipitated by making the solution 1 M with respect to sodium chloride and leaving it at 0° for 18 hours. The ribonucleates were recovered and reprecipitated twice more from sodium chloride solution without drying between precipitations. The final precipitate was washed and dried with ethanol and ether as described above and the final yield from 90 grams of wheat germ was about 1 gram of a white powder with flour-like consistency. The ribonucleates obtained in this way had an extinction coefficient (260 mμ) of 220-250 when dissolved in distilled water to give a 1% solution, and were, therefore, 95 - 100% pure.

(ii) Preparation of phosphodiesterase from Russell Viper venom.

The following procedure has been designed to recover the phosphodiesterase activity of whole venom devoid of the phosphomonoesterase and nuclease activities which are also present in the whole venom. Lyophilized or sublimated Russell Viper venom was purchased from Ross Allen's Reptile Institute and first subjected to acetone fractionation according to the procedure of Koerner and Sinsheimer (1957). Phosphodiesterase activity was assayed as described in Part II (i) of the thesis. Assay of phosphomonoesterase activity of whole Russell Viper venom was performed by measuring the release of inorganic phos-

phate under the conditions used for phosphodiesterase assay except that the ribonucleates were replaced by an equal weight of 5'-adenylate as substrate. The cleavage of monoester phosphate by whole venom was about seven times as great as the cleavage of diester phosphate on a molar basis.

The entire acetone fractionation procedure was carried out at 0°. Two hundred mg of the venom were dissolved in 12 ml of cold distilled water and the solution was stirred for 2 min prior to the addition of 8 ml of 0.5 M ammonium acetate buffer, pH 4 (see Appendix). After 2 min of stirring, 14.5 ml of acetone were added dropwise with stirring, and then stirring was continued for one hour. The precipitate was removed by centrifugation at 4400 g for 15 min. The clear supernatant solution was mixed with 5.5 ml of acetone by dropwise addition, with stirring, and, after stirring for a further 15 min, the precipitate was collected by centrifugation at 4400 g for 15 min. Excess acetone was allowed to evaporate by leaving the pellet in air for two min. The pellets obtained in two such preparations were dissolved in 50 ml of distilled water.

The solution of phosphodiesterase recovered from acetone fractionation was further purified by a modification of the original procedure of Hurst and Butler (1951). The solution was passed through a 5 cm x 2.5 cm cellulose column prepared from 5 gm of Whatman purified cellulose pulp and the effluent was collected in

TABLE I

The purity and activity of snake venom phosphodiesterase at various stages in the purification procedure.

Purification step	s.u. (280 mμ)	Volume (ml)	Volume assayed (ml)	Assay activity*	Total activity	Specific activity**
Whole venom	476	24	0.021	6.55	7,486	16.0
After acetone fractionation	110	11	0.05	20.8	4,576	41.6
After 5 gm cellulose column	67	70	0.1	6.04	4,228	61.5
After 7 1-gm cellulose columns	41	170	0.5	6.60	2,244	54.7
After 4 1-gm cellulose columns	26	110	-	-	-	-
After 3 1-gm cellulose columns	17	62.5	0.5	9.36	1,264	74.4

* μmole phosphodiester linkages cleaved per hour in the standard assay.

** μmole phosphodiester linkages cleaved per hour per mg protein in the standard assay. (1 s.u. (280 mμ) is presumed to correspond to 1 mg of protein).

10 ml fractions. Distilled water was passed through the column until the optical density (280 mμ) of the effluent decreased to zero after collecting about twelve fractions. The ultraviolet absorption spectrum of each of the fractions indicated that acetone from previous purification steps was present and thus the pooled fractions were evaporated in a flash evaporator at 25° to a volume of about 40 ml in order to remove the acetone, and the resulting solution was then diluted with distilled water to give an optical density of 0.5 at 280 mμ. The volume of the enzyme solution at this stage was about 120 ml. Aliquots of 20 ml were passed through 1 cm x 2.5 cm cellulose columns prepared from 1 gm of Whatman purified cellulose pulp. Distilled water was then passed through the columns until the optical density (280 mμ) of the effluents decreased to zero. Evaporation and fractionation of the pooled effluents on 1 gm cellulose columns were continued under the conditions just described, until the pooled effluents contained about 15 mg of protein (15 s.u. measured at 280 mμ). The resulting solution had suitable phosphodiesterase activity and was usually devoid of the contaminating phosphomonoesterase or nuclease activities present in the original venom. The details of a typical purification, conducted on 400 mg of Russell Viper venom, are shown in Table I.

(iii) Preparation of pNp compounds.

Five hundred mg of high molecular weight ribonucle-

ates isolated from E. coli were dissolved in 25 ml of distilled water. The ribonucleate solution was mixed with 25 ml of 2 M ammonium hydroxide solution, adjusted to pH 10 with concentrated formic acid, and the resulting solution was incubated at 51° for 18 hours. The pH of the solution was then adjusted to 9.2 by the addition of concentrated formic acid before the addition of 20 mg of lyophilized Russell Viper venom. Digestion with whole venom was allowed to proceed at 37° for 6 hours and then the solution was mixed with 10 ml of a 10 M potassium hydroxide solution. The alkali hydrolysis was allowed to proceed at room temperature for 18 hours. The solution was then cooled to 0° and neutralized with concentrated perchloric acid. The copious precipitate of potassium perchlorate was removed by centrifugation and further precipitation of salt was effected by placing the supernatant solution at 0° for one hour. The clear supernatant solution was decanted and diluted to two litres with distilled water and the resulting solution was passed into a 20 cm x 2.5 cm column of DEAE-cellulose (in the formate form). Nucleosides and nucleoside monophosphates were eluted from the column as described in Part II (ii) of the thesis. The column was washed with one litre of water and then pNp compounds were eluted with 100 ml of 1 M pyridinium formate, pH 4.5. The pyridinium formate was evaporated and the residue dissolved in 25 ml of 0.6 M ammonia. The ammonia solution was evaporated and the resulting residue was dissolved in 10 ml of distilled water. The solution

contained 1300 s.u. of nucleotide material (measured at 260 mμ). The pNp compounds were contaminated with a large quantity of alkali-labile oligonucleotides at this stage, and thus the solution was made molar with respect to potassium hydroxide and incubated for 90 hours at room temperature in order to convert the oligonucleotides to mononucleotides. The hydrolyzate was neutralized and fractionated on a DEAE-cellulose column as described in Part II (ii) of the thesis. The final yield of pure pNp compounds was 325 s.u. (260 mμ), and their purity was established by two-dimensional chromatographic analysis using system A and system B (see Part II (iv)) in succession. The studies of Part V (iv) of the thesis have suggested simpler procedures for preparing these compounds in improved yields but the foregoing description is a detailed account of the origin of the pNp compounds which were examined in Part IV (i) of this thesis.

(iv) Preparation of pN>p compounds.

Five hundred mg of high molecular weight ribonucleates isolated from E. coli were dissolved in 50 ml of distilled water and the resulting solution was mixed with 15 ml of 1 M ammonium formate buffer, pH 9.2 before the addition of 8 ml of a solution containing 8 mg of Russell Viper venom and 1.25γ of crystalline pancreatic ribonuclease. The resulting solution was incubated at 37° for 20 hours before dilution with water to 666 ml, and after adjusting the pH of the diluted hydrolyzate to 7.8, the sol-

ution was passed into a 15.5 cm x 2.5 cm column of DEAE-cellulose pre-equilibriated with 0.025 M TRIS-formate, pH 7.8. Nucleosides and nucleoside monophosphates were eluted from the column as described in Part II (ii) of the thesis, before washing the column with 500 ml of distilled water. The pN>p compounds were eluted from the column with 130 ml of 1 M pyridinium formate, pH 4.5 and were recovered and chromatographed as described in section (iii) of this Part of the thesis. The final yield of pure pN>p compounds was 935 s.u. (260 mμ).

PART II - ANALYTICAL PROCEDURES

(i) Assay of snake venom phosphodiesterase activity.

The activity of phosphodiesterase preparations was assayed by measuring the rate of release of acid-soluble material during the hydrolysis of ribonucleates. The following solutions were mixed in rapid succession with the aid of a vortex mixer: 1 ml of a 1% aqueous solution of RNA (10 mg, 250 s.u., 23 μm of constituent nucleotides), 0.5 ml of 1 M ammonium formate buffer, pH 9.2 (500 μm ammonia, 500 μm ammonium formate) and 0.5 ml of enzyme solution (150 γ protein for "purified" enzyme, 1000 γ for crude venom). The resulting solution was incubated at 37° for one hour and then mixed, using a vortex mixer, with 4 ml of a 5% aqueous solution of TCA (0.3 M, 1200 μm TCA). The resulting precipitate was removed by centrifugation and the absorbance of the supernatant solution was measured at 260 mμ. A 20-fold dilution of the supernatant solution was usually required for measurements of optical density. The total s.u. (260 mμ) in the supernatant solution was then calculated:

volume supernatant solution x dilution x absorbance (260 mμ)
or: 5.8 x 20 x A_{260}

A correction for the contribution of reagents to the total absorbance of supernatant solutions following TCA precipitation was obtained by performing the assay with 0.5 ml of distilled water rather than enzyme solution. This correction amounted to 4 s.u. and was subtracted from the assay

values obtained with enzyme solutions.

For complete hydrolysis of 10 mg of ribonucleates in one hour, the supernatant solution after TCA precipitation contained about 254 s.u. Application of the correction gave a value of 250 s.u. for the amount of nucleotide material in the supernatant solution after complete hydrolysis. Assays were performed with phosphodiesterase solutions which had a range of activity corresponding to the release of 70 - 90 s.u. of TCA-soluble material in the assay (about 30 - 40% hydrolysis).

The phosphodiesterase activity of solutions in this standard assay could be conveniently expressed as a hydrolysis rate (hr^{-1}) in two different ways.

(i) $$\% \text{ hydrolysis} = \frac{\text{s.u. in TCA supernatant solution} - 4}{250} \times 100$$

(ii) µmoles of phosphodiester linkages cleaved

$$= \frac{\text{s.u. in TCA supernatant solution} - 4}{11}$$

The mean mmolar extinction coefficient (260 mµ) of the four major nucleotide components of the ribonucleates is 11, and for 100% cleavage the hydrolysis rate was $\frac{250}{11}$ = 23 µm of phosphodiester linkages cleaved per hour. The second mode of expressing activity is valid since phosphodiesterase is an exonuclease which releases one mole of 5'-mononucleotide per mole of phosphodiester linkage cleaved, and the mononucleotides are completely soluble in TCA while polymers greater than about ten nucleotides in length are not soluble in TCA. Therefore the correlation between

μmoles of phosphodiester linkages cleaved and TCA-soluble nucleotides is valid even after 99% hydrolysis of the ribonucleates.

(ii) Anion-exchange fractionation of alkali and phosphodiesterase hydrolyzates on DEAE-cellulose.

Standard DEAE-cellulose # 70 was purchased from Carl Schleicher and Schuell Co. and was converted to the formate form by the following procedure. About 100 gm of DEAE-cellulose was suspended in 2 litres of 1 M KOH and the suspension was stirred for 5 minutes prior to filtration on a sintered-glass funnel. The cake obtained after filtration was washed with 1 M ammonium formate buffer, pH 5.4 until the effluent had a pH value of 5.4 whereafter the DEAE-cellulose was washed with 0.025 M ammonium formate buffer, pH 5.4 until the effluent had an optical density at 220 mμ of about 0.4 and an optical density at 260 mμ of zero.

Alkali and phosphodiesterase hydrolyzates of ribonucleates were neutralized with formic acid and diluted to give a salt concentration of about 0.02 M. Alkali hydrolyzates containing between 10,000 and 25,000 s.u. (260 mμ) were passed into 12 cm x 4.5 cm DEAE-cellulose columns. Phosphodiesterase hydrolyzates containing 1,300 s.u. (260 mμ) were passed into 7 cm x 2.5 cm DEAE-cellulose columns. The compounds present in the hydrolyzates were eluted from the columns in the order of increasing negative charge of the compounds. At pH 7.8, nucleosides (N) have zero charge, nucleoside monophosphates (pN and Np) have two negative

TABLE II

Eluents used to fractionate alkali hydrolyzates of the 18S + 28S ribonucleates on DEAE-cellulose.

Compound:	Net charge at pH 7.8	Eluent:
N	0	H_2O
Np	-2	0.085 *M* TRIS formate, pH 7.8, 7 *M* Urea
NxpNp	-3	0.17 *M* TRIS formate, pH 7.8, 7 *M* Urea
pNp + NxpNxpNp	-4	1 *M* Pyridinium formate, pH 4.5

TABLE III

Eluents used to fractionate phosphodiesterase hydrolyzates of the 18S + 28S ribonucleates on DEAE-cellulose.

Compound:	Net charge at pH 7.8	Eluent:
N	0	H_2O
pN	-2	0.085 *M* TRIS formate, pH 7.8, 7 *M* Urea
pN>p + pNpN	-3	0.35 *M* TRIS formate, pH 7.8, 7 *M* Urea
pNp + pNpNpN	-4	
$(pN)_n$, n>3	<-4	1 *M* Pyridinium formate, pH 4.5

charges, dinucleotides (pNpN and NxpNp) and cyclic nucleoside diphosphates (pN>p) have three negative charges, while open nucleoside diphosphates (pNp) and trinucleotides (pNpNpN and NxpNxpNp) have four negative charges. The eluents used for stepwise elution of these compounds are listed in Tables II and III. It is necessary to include urea in the eluents in order to achieve separation on the basis of net charge (Tomlinson and Tener, 1963).

(iii) Desalting of nucleate derivatives in the effluents from DEAE-cellulose columns.

The nucleate derivatives in effluents obtained from the fractionation of hydrolyzates of ribonucleates on DEAE-cellulose were desalted by adsorption to and elution from either charcoal or DEAE-cellulose.

The pH value of the effluent containing nucleosides (0.1 - 1 μmoles in 200 - 300 ml of solution) was adjusted to 6 by the addition of a few drops of concentrated formic acid and the resulting solution was then passed through a 50 mg charcoal disc which was mounted between two layers of # 545 celite in a column which was 2.5 cm in diameter. The column was then washed with 150 ml of distilled water to remove residual salt, and the nucleosides were eluted from the charcoal with an eluent made by mixing 45 ml of distilled water, 45 ml of 95% ethanol and 4 ml of concentrated ammonia. The ammoniacal ethanol effluent was evaporated in a flash evaporator at 40° and the resulting dry residue was recovered in a volume of 0.1 - 0.2 ml of water for paper chromatography. The recovery of nucleosides by

TABLE IV

The desalting of effluents from DEAE-cellulose columns by adsorption to and elution from charcoal.

Compound:	Charge** (s.u.)	Weight of charcoal	No. of discs	Recovery (%)			
				a*	g*	c*	u*
Nucleosides (N)	13.6	50 mg	1	82	86	86	90
"	13.0	"	1	85	85	90	93
"	7.1	"	1	80	86	92	87
"	2.9	"	1	84	90	80	100
"	1.7	"	1	80	83	100	100
" ***	13.0	"	1	85	85	90	93
Nucleotides (Np)	10.5	"	1	86	82	87	86
Nucleoside diphosphates (pNp)	12.1	"	1	72	-	64	62
"	"	200 mg	1	72	-	64	62
"	"	50 mg	2	74	-	75	72
"	"	"	4	80	-	81	74
" ***	"	"	1	48	-	20	20

* The constituent base of the compounds listed in column 1.

** The compounds were charged from 250 ml of 0.05 M ammonium formate, pH 5.4 and washed with 100 ml of water.

*** The compounds were charged from salt-free solutions.

this desalting procedure was about 85 per cent.

Nucleoside monophosphates could be effectively desalted in the same manner, with about 85 per cent recovery, but nucleoside diphosphates could only be recovered in comparable yields by using elaborately constructed columns having multiple layers of charcoal. A summary of the results obtained with the different types of compounds is shown in Table IV. It is particularly noteworthy that the pNp compounds adsorbed to charcoal very poorly from salt-free solutions in contrast with the nucleosides and nucleoside monophosphates which seemed to be unaffected by the presence or absence of salt in the influent.

The compounds having phosphate groups (pN, pNp and pN>p) were usually desalted on DEAE-cellulose columns in the following manner. The effluents containing 1 - 100 s.u. of the phosphorylated compounds were diluted with distilled water to a salt concentration of about 0.03 M and were then passed into 4 cm x 2.5 cm columns of DEAE-cellulose. The columns were washed extensively with distilled water (1 - 1.5 litres) to remove residual salt and urea, and then the nucleotides were eluted from the cellulose with 100 ml of 1 M pyridinium formate, pH 4.5. The pyridinium formate, being a volatile salt, was removed by evaporation in a flash evaporator at 40° and the pyridinium salts of the nucleotides were converted to ammonium salts by evaporation in 0.5 M ammonium hydroxide solution. The recoveries of nucleotides were about 90 per cent.

Any oligonucleotide material in phosphodiesterase

hydrolyzates was eluted from DEAE-cellulose by 1 M pyridinium formate and the effluent was desalted by direct evaporation at 40°. The oligonucleotides were converted to their ammonium salts by evaporation in 0.5 M ammonium hydroxide solution.

(iv) Separation of N, pNp and pN>p compounds by paper chromatography.

The nucleosides and nucleoside diphosphates were separated by paper chromatography. The aqueous solutions of nucleosides and nucleoside diphosphates from column separations on DEAE-cellulose were desalted and concentrated for spotting as described in Part II (iii) of the thesis. The solvent for one-dimensional separations was composed of 80 volumes of 95% ethanol and 20 volumes of distilled water and was used together with Whatman No. 1 paper which had been impregnated with ammonium sulphate by dipping through a solution made by mixing 10 volumes of saturated ammonium sulphate solution with 90 volumes of distilled water (Lane, 1963). This will be subsequently referred to in the thesis as system A. When separation of pNp and pN>p compounds was incomplete in a one-dimensional chromatogram, further separation was achieved with a second dimensional development using a solvent made by mixing 80 volumes of saturated ammonium sulphate solution and 2 volumes of isopropanol. This will be subsequently referred to in the thesis as system B.

One-dimensional chromatography was always satisfac-

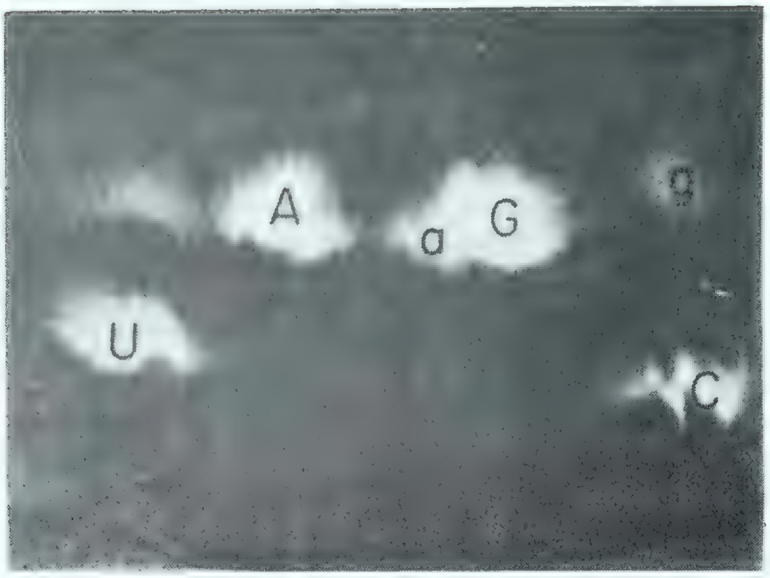
tory for resolving the nucleosides of alkali or phosphodiesterase hydrolyzates, and was usually adequate for resolving the nucleoside diphosphates of phosphodiesterase hydrolyzates. Two-dimensional chromatography was essential for separating the nucleoside diphosphates of alkali hydrolyzates from alkali-stable trinucleotides.

TABLE V

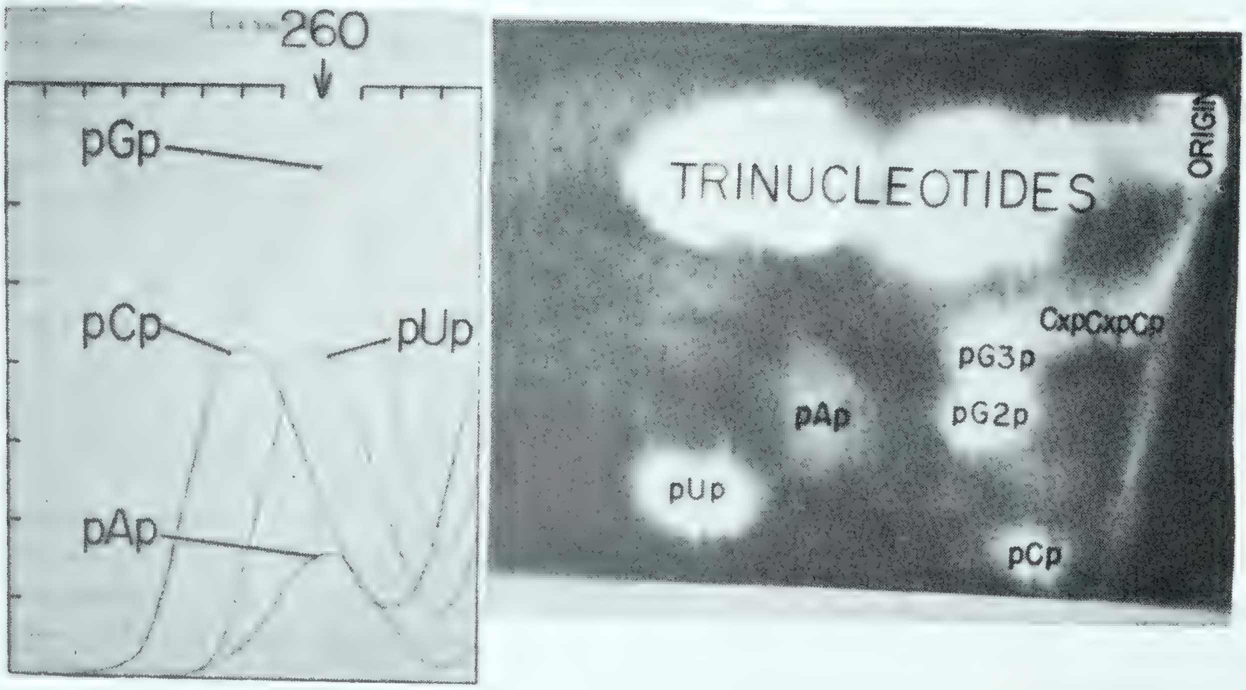
The fractionation on DEAE-cellulose of a 90 hour alkali hydrolyzate of 18S + 28S ribonucleates from wheat germ

Original charge	27,600 s.u.
N	36 s.u.
Np	26,442 s.u.
NxpNp	1,025 s.u.
pNp + NxpNxpNp	136 s.u.
Total recovery	27,639 s.u.

Nucleosides



Ultraviolet photographs of two-dimensional paper chromatograms which depict the separation of nucleosides and diphosphonucleosides derived from 90 hour alkali hydrolyzates of the 18S + 28 ribonucleates from wheat germ.



Diphosphonucleosides

Figure 1

PART III - ANALYSIS OF THE NATIVE END GROUPS OF 18S + 28S RIBONUCLEATES FROM WHEAT GERM.

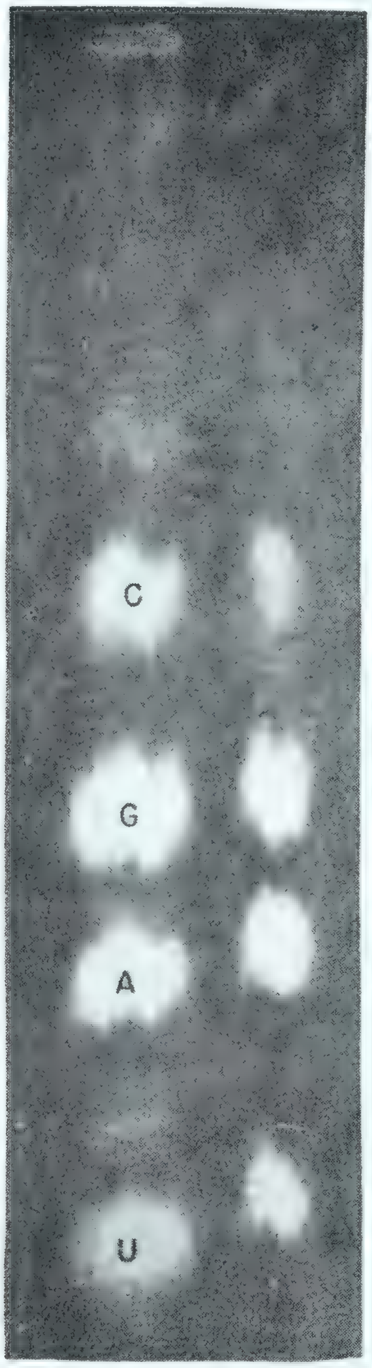
(i) The rational and experimental basis of the analysis.

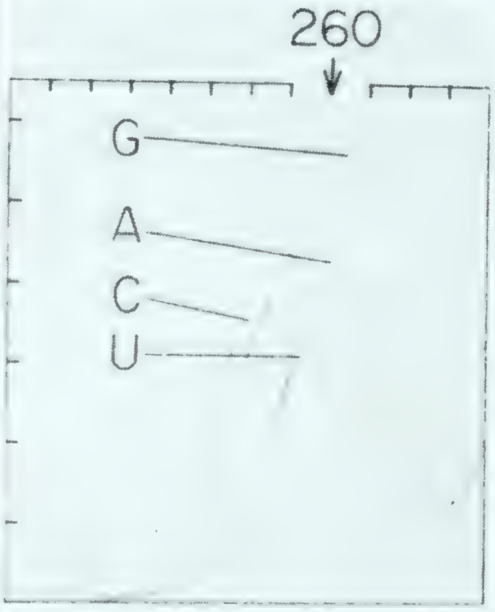
The rational basis for the view that the native end groups of the 18S + 28S ribonucleates appear as nucleosides and nucleoside diphosphates in alkali hydrolyzates has been discussed in the Introduction of the thesis.

The experimental basis for the column fractionation of alkali hydrolyzates, and for the recovery of nucleosides and nucleoside diphosphates from column fractions, has been described in Part II (ii) and Part II (iii) of the thesis, respectively.

The details for one such column fractionation of a 90 hour alkali hydrolyzate of the 18S + 28S wheat germ ribonucleates are shown in Table V. The two-dimensional paper chromatographic separations of the nucleosides and nucleoside diphosphates from the 90 hour alkali hydrolyzate described in Table V are illustrated by the ultraviolet photographs of Figure 1. The ultraviolet spectra of the nucleoside diphosphates are also shown in Figure 1. A double-wavelength calculation is needed for the quantitative measurement of guanosine in the nucleoside fraction of 90 hour hydrolyzates because of the presence of adenine which separates imperfectly even after two-dimensional chromatography. The bases result from trace N-glycosyl cleavage during the extensive treatment in 1 M alkali. The ultraviolet photograph and spectral chart

Ultraviolet photograph of a one-dimensional paper chromatogram depicting the separation of the nucleosides obtained from a 43-hour alkal hydrolyzate of the 18S + 28S ribonucleates from wheat germ.





Spectral chart

Ultraviolet photograph

Figure 2

of Figure 2 show the one-dimensional separation and spectral analysis of nucleosides from a 43 hour alkali hydrolyzate, and it is evident from the guanosine spectrum that contamination by adenine is negligible.

The processing of the primary data will be outlined for the nucleoside diphosphates of the 90 hour alkali hydrolyzate and for the nucleosides of the 43 hour alkali hydrolyzate.

The aliquot of pNp compounds used for paper chromatography (Figure 1) corresponded to 40% of the material in the pNp + NxpNxpNp fraction (Table V), and each compound was eluted from the chromatogram in 5 ml of 0.1 M hydrochloric acid solution. The total amount of nucleotide material in the hydrolyzate from which the pNp compounds were isolated was $\frac{27,600}{11} = 2,510$ μmoles, and the overall recovery of pNp compounds was presumed to be 90 per cent. Thus, the quantity of each nucleoside diphosphate in mole per 100 moles of total nucleotides was estimated in the following manner:

$$\text{pAp:} \quad 0.160 \times 5 \times \frac{1}{14.2} \times \frac{100}{2510} \times \frac{100}{40} \times \frac{100}{90} = 0.006$$

$$\text{pGp:} \quad 0.650 \times 5 \times \frac{1}{11.6} \times \frac{100}{2510} \times \frac{100}{40} \times \frac{100}{90} = 0.031$$

$$\text{pCp:} \quad 0.230 \times 5 \times \frac{1}{6.4} \times \frac{100}{2510} \times \frac{100}{40} \times \frac{100}{90} = 0.020$$

$$\text{pUp:} \quad 0.419 \times 5 \times \frac{1}{10} \times \frac{100}{2510} \times \frac{100}{40} \times \frac{100}{90} = 0.023$$

Net pNp = 0.080 mole/100 moles nucleotides.

The aliquot of N compounds used for paper chromato-

TABLE VI

The end groups determined by alkali hydrolysis of the 18S + 28S ribonucleates from wheat germ.

Component	mole/100 moles nucleotides	
	Preparation 1	Preparation 2
A	0.012	0.016
G	0.024	0.024
C	0.021	0.019
U	0.021	0.019
Total	0.078	0.078
pAp	0.008	0.007
pGp	0.031	0.032
pCp	0.020	0.018
pUp	0.023	0.020
Total	0.082	0.077

TABLE VII

The nucleosides and nucleoside diphosphates determined after alkali hydrolysis of degraded ribonucleate preparations.

Preparation of	mole/100 moles nucleotides									
ribonucleates:	A	G	C	U	Total N	pAp	pGp	pCp	pUp	Total pNp
Commercial ribonucleates	0.22	0.19	0.11	0.17	0.69*	-	-	-	-	-
E. coli ribonucleates	0.036	0.007	0.010	0.023	0.076**	0.019	0.020	0.002	0.010	0.051*
	±0.002	0.001	0.002	0.001	0.004					

* This is the result of a single analysis.

** These data summarize the results from three determinations.

graphy (Figure 2) corresponded to all of the material in the nucleoside fraction of a 43 hour hydrolyzate and each compound was eluted in 5 ml of water. The total amount of material in the hydrolyzate from which the nucleosides were isolated was $\frac{10,890}{11}$ = 990 μmoles, and the overall recovery of nucleosides was presumed to be 85 per cent. Thus, the quantity of each nucleoside in mole per 100 moles of total nucleotides was estimated in the following manner:

A: $0.437 \times 5 \times \frac{1}{15} \times \frac{100}{990} \times \frac{100}{85} = 0.016$ mole/100 moles

G: $0.508 \times 5 \times \frac{1}{11.8} \times \frac{100}{990} \times \frac{100}{85} = 0.026$ "

C: $0.258 \times 5 \times \frac{1}{7.5} \times \frac{100}{990} \times \frac{100}{85} = 0.020$ "

U: $0.338 \times 5 \times \frac{1}{10} \times \frac{100}{990} \times \frac{100}{85} = 0.020$ "

Net N = 0.082 mole/100 moles nucleotides.

A summary of the results of nucleoside and nucleoside diphosphate analyses on two preparations of 18S + 28S wheat germ ribonucleates is shown in Table VI.

The data of Table VII show the results of similar analyses performed on degraded ribonucleate preparations. The analysis of the "8S ribonucleates" from *E. coli* was performed on the same scale as the analysis shown in Table V but the analysis of the commercial specimen was performed on only 800 s.u. of material. The lack of equivalence between the quantity of nucleosides and the quantity of nucleoside diphosphates in the case of the fragmented ribonucleates from *E. coli* probably relates to the fact

that only one attempt was made to analyze the nucleoside diphosphates, and in this attempt, all materials binding to DEAE-cellulose more tightly than the 2' and 3' nucleotides were eluted with 1 M pyridinium formate. About 50 per cent of the material binding more tightly than 2' and 3' nucleotides was not nucleoside diphosphate and interfered with the resolution of nucleoside diphosphates. Consequently the presumption of 90 per cent recovery is, in this instance, almost certainly an underestimate.

(ii) The significance of the results for concepts of the chemical structure of 18S + 28S ribonucleates.

The data on the type I structures present in degraded ribonucleate preparations show that limited degradation, as in the case of the *E. coli* ribonucleates, does not result in any notable increase in the end groups of type I structures. The studies in Part IV (ii) of this thesis show that the end groups resulting from limited fragmentation are characteristic of type II and III structures in the case of the *E. coli* ribonucleates. Even after extensive degradation, as in the case of the commercial ribonucleate sample, it is clear that the increased quantity of end groups of type I structures is about tenfold smaller than the increase in the quantity of the end groups of type II and III structures (see Part IV (ii)).

The data on the 18S + 28S ribonucleates from wheat germ indicate that the primary structures have a mean chain length of about 1300 nucleotide residues, and the

presence of all four bases at each type of chain terminus shows that there are, minimally, four different chains. The possibility that the 28S component may be composed of chains having the same length and end group characteristics as the 18S chains arises from the fact that the number average molecular weight of the 18S + 28S ribonucleates, by end group analysis, is consonant with the physicochemical estimates of the weight average molecular weight for the 18S component.

Further speculation would not seem warranted but these data have obvious relevance to current concepts of ribonucleate biosynthesis. They should assist in discerning among different possible mechanisms which are being put forth concerning chain initiation, termination and cleavage processes associated with ribonucleate biosynthesis.

PART IV - ANALYSIS OF THE END GROUPS WHICH RESULT FROM PHOSPHODIESTER CLEAVAGE OF RIBONUCLEATE CHAINS.

(i) Hydrolysis of ribonucleates by snake venom phosphodiesterase.

(a) The rational and experimental basis for the method.

The rational basis for the view that end groups produced by ribonucleate cleavage appear as nucleosides and diphosphonucleosides in phosphodiesterase hydrolyzates has been described in section (ii) of the Introduction to this thesis.

The experimental conditions for the end group analysis with phosphodiesterase were identical with the conditions described for assay of the enzyme (see Part II (i)) except that the hydrolysis was performed on a five-fold larger scale, in order to obtain measurable quantities of end groups. Thus 50 mg of ribonucleates in 5 ml of water, was mixed with 2.5 ml of 1 M ammonium formate buffer, pH 9.2 and 2.5 ml of purified enzyme. The enzyme hydrolyzed the ribonucleates at a rate of 7 μmoles of phosphodiester linkages per hour in the standard assay, corresponding to a rate of 5 x 7 = 35 μmoles of phosphodiester linkages in the first hour of the end group analysis. The mixture was incubated at 37° for 24 hours before diluting to 100 ml with distilled water, and adjusting the pH to 6.5 with 1.5 M acetic acid. The optical density was measured and the hydrolyzate was passed into a column of DEAE-cellulose and fractionated as described in Part II (ii) of the thesis.

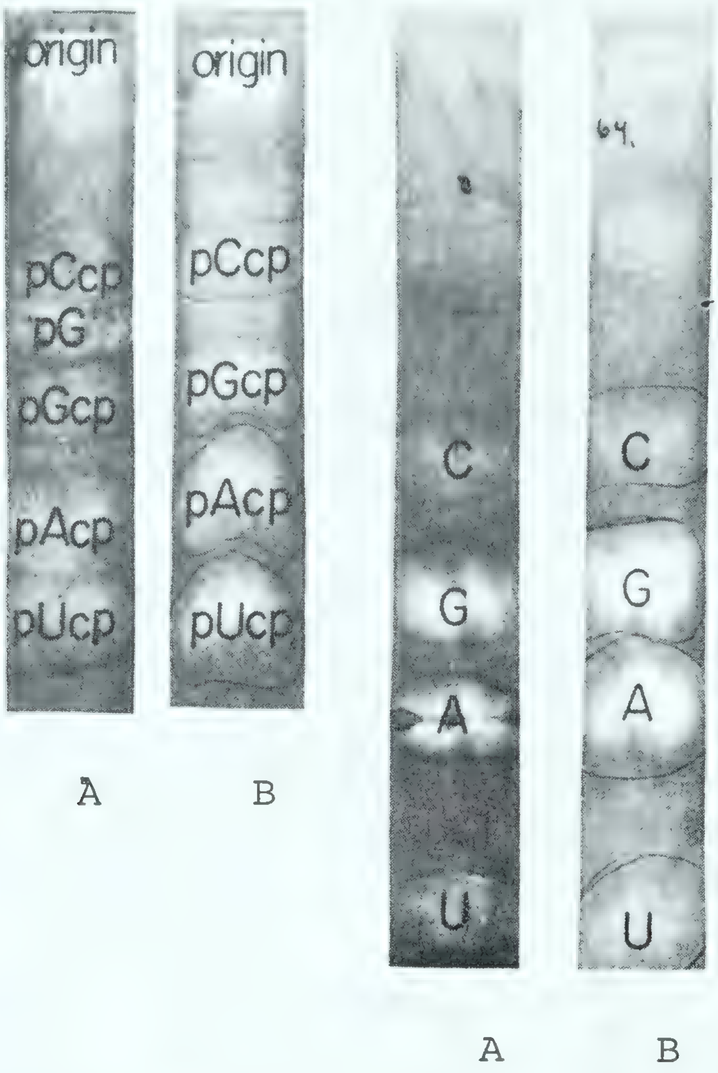
TABLE VIII

Results of column fractionation on DEAE-cellulose of a typical phosphodiesterase hydrolyzate.

Fraction	s.u. (260 mμ)	Volume of eluent (ml)
Original charge	1318	-
N	1.9	250
pN	1309	885
pN>p	2.9	175
Oligonucleotide	5.5*	100

* Spectral analysis showed this to be largely non-nucleotide material.

Paper chromatographic resolution and spectral analysis of the nucleosides and diphosphonucleosides derived from phosphodiesterase hydrolyzates of the 18S + 28S ribonucleates from wheat germ.



A: an analysis yielding about 0.1 mole per cent of each of N and pN>p.

B: an analysis yielding about 0.2 mole per cent of each of N and pN>p.

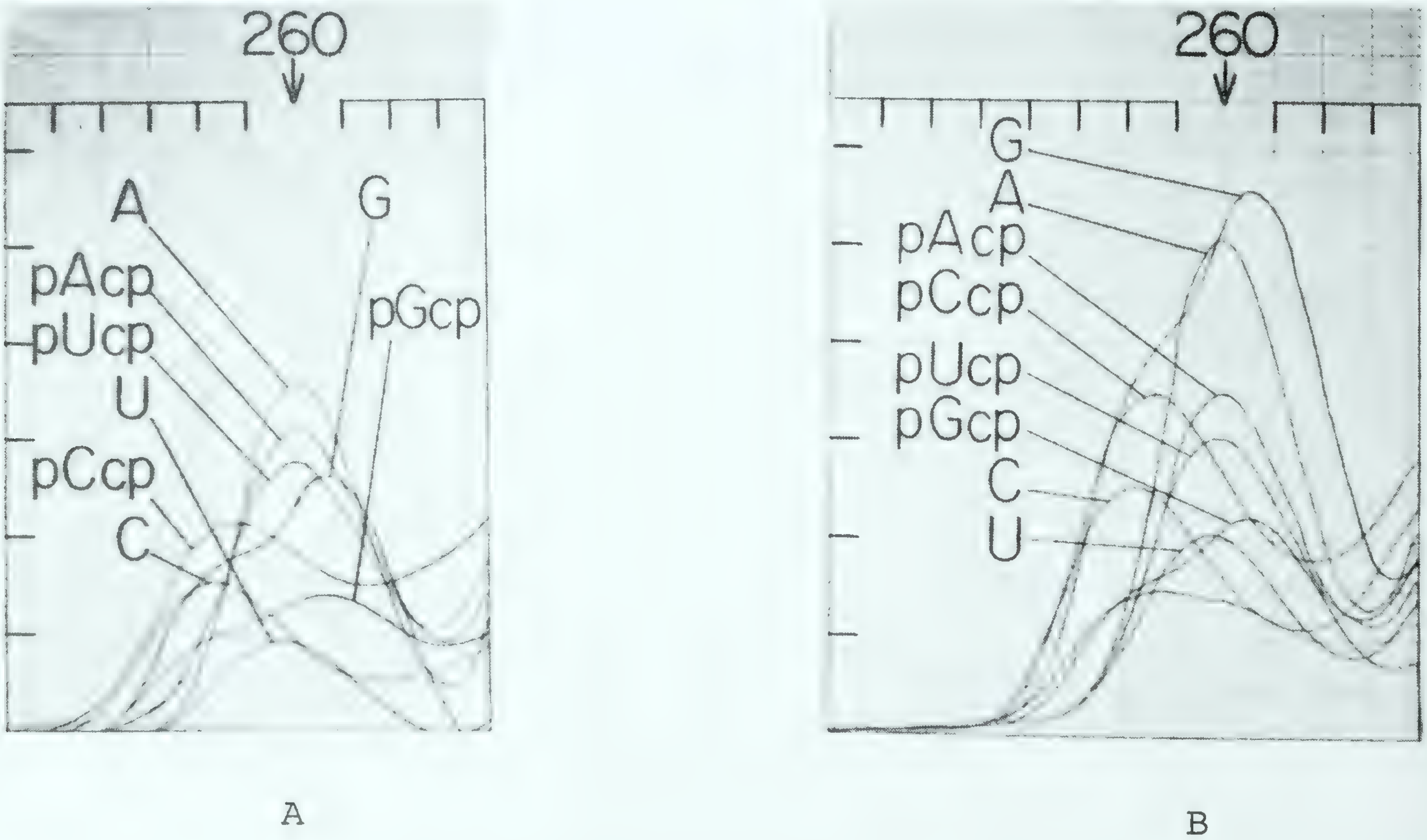


Figure 3.

The resulting fractions were desalted and chromatographed on paper as described in Part II (iii) and Part II (iv), respectively.

In a typical analysis, 50 mg of the 18S + 28S ribonucleates isolated from wheat germ gave an original charge of 1318 s.u. of nucleotide material after hydrolysis with phosphodiesterase. A balance sheet for the column fractionation of the hydrolyzate on DEAE-cellulose is shown in Table VIII. The amounts of the individual components in the nucleoside and diphosphonucleoside fractions were calculated after separation by paper chromatography in system A. The individual compounds were eluted from the paper with 1.5 ml of distilled water, and characterized spectrally between 220 mμ and 340 mμ using a Bausch and Lomb spectrophotometer. The quantities of individual compounds were calculated and expressed as mole per 100 moles of total nucleotides in the phosphodiesterase hydrolyzates with due regard for the 85 per cent recovery of nucleosides and 90 per cent recovery of diphosphonucleosides. Typical separations are shown in Figure 3 together with the spectra of the compounds after elution from the chromatograms. The calculations shown below specifically apply to the hydrolyzate summarized in Table VIII and documented in the spectral chart shown in the left hand side of Figure 3.

Calculations:

<u>Nucleosides:</u>

A: $0.358 \times 1.5 \times \frac{1}{15} \times \frac{100}{120} \times \frac{100}{85} = .035$

G: $0.233 \times 1.5 \times \frac{1}{11.6} \times \frac{100}{120} \times \frac{100}{85} = .030$

TABLE IX

Results of end group analyses performed with snake venom phosphodiesterase preparations contaminated by nuclease or phosphomonoesterase activities.

Expt.	Substrate concentration	Enzyme activity**	mole/100 moles nucleotides					% Oligonucleotide residue
			A	G	C	U	Total N	
1a	0.5%	35	1.26	0.071	0.059	0.059	1.45	0
1b*	0.5%	35	1.29	0.076	0.059	0.065	1.49	0
2a	0.5%	35	0.32	0.048	0.045	0.025	0.44	0
2b	0.25%	35	0.078	0.040	0.020	0.022	0.17	0
3a	0.5%	29	1.02	1.14	0.25	0.53	2.94	0.3
3b*	0.5%	29	2.38	2.88	0.51	1.16	6.93	0

* Incubation was extended to 48 hours with an addition of fresh enzyme at 24 hours. The additional enzyme was equivalent to the quantity added initially to achieve complete hydrolysis in 24 hours.

** μmoles of phosphodiester linkages cleaved in the first hour of the end group analysis.

C: $0.091 \times 1.5 \times \frac{1}{7} \times \frac{100}{120} \times \frac{100}{85} = .019$

U: $0.091 \times 1.5 \times \frac{1}{10} \times \frac{100}{120} \times \frac{100}{85} = .013$

Net N = .097 mole/100 moles nucleotide material present in the hydrolyzate.

Diphosphonucleosides:

pA>p: $0.309 \times 1.5 \times \frac{1}{15} \times \frac{100}{120} \times \frac{100}{90} = 0.029$

pG>p: $0.132 \times 1.5 \times \frac{1}{11.6} \times \frac{100}{120} \times \frac{100}{90} = 0.016$

pC>p: $0.182 \times 1.5 \times \frac{1}{7} \times \frac{100}{120} \times \frac{100}{90} = 0.024$

pU>p: $0.277 \times 1.5 \times \frac{1}{10} \times \frac{100}{120} \times \frac{100}{90} = 0.038$

Net pN>p = .107 mole/100 moles nucleotide material present in the hydrolyzate.

(b) The effects of nuclease and phosphomonoesterase contamination of snake venom phosphodiesterase preparations.

Preparations of snake venom phosphodiesterase which were contaminated with nuclease or phosphomonoesterase yielded spuriously high end group values.

Results of analyses in which contaminated enzymes were used in analyses for nucleoside end groups are tabulated in Table IX. These data have been discussed at some length elsewhere. (Lane, Diemer and Blashko, 1963).

(c) An unusual side-reaction with amino alcohol buffers.

The employment of amino alcohol buffers such as TRIS, DIOL and aminoethanol, for buffering phosphodiesterase hydrolyzates, results in the formation of phosphodiesters be-

TABLE X

The yield of nucleosides and unusual phosphodiester derivatives formed during end group analyses in which amino alcohol buffers were employed.*

pH	Buffer**	mole/100 moles nucleotides					% Oligonucleotide residue	mole/100 moles nucleotides				
		A	G	C	U	Total N		BpA	BpG	BpC	BpU	Total BpN
8.0	TF	.019	-	-	-	0.019	75	.14	.12	.092	.074	0.43
8.6	DF	.055	.074	.033	.020	0.18	0	.024	.035	.039	.021	0.12
9.2	AF	.071	.044	.025	.028	0.17	18	.00	.00	.00	.00	0.00
9.5	EF	.087	.089	.048	.052	0.28	25	.035	.038	.024	.020	0.12

* The activity of the enzyme, assayed at pH 9.2, was 22 in the experiments at pH values 8.0, 9.2 and 9.5, and was 50 in the experiment at pH 8.6. The enzyme activity is expressed as μmoles of phosphodiester linkages cleaved by the enzyme in the first hour of an end group analysis at pH 9.2.

** TF - TRIS formate (0.25 M); DF - DIOL formate (0.25 M); AF - Ammonium formate (0.25 M); EF - Ethanolamine formate (0.25 M).

TABLE XI

Results of a typical experiment to determine the stability of pNp compounds in phosphodiesterase hydrolyzates.*

Time (hrs)	μmole									
	pAp	pGp	pCp	pUp	Total	pA	pG	pC	pU	Total
24	0.058	0.072	0.059	0.082	0.27	0.21	0.089	0.17	0.21	0.68

* The conditions were precisely the same as those of a standard end group analysis except that the ribonucleates were replaced by pNp compounds (see text). The recovery after desalting and one-dimensional chromatography was about 90 per cent.

TABLE XII

The effect of substrate concentration, product concentration and phosphodiesterase inhibitors on the hydrolysis of pNp compounds in the presence of phosphodiesterase.*

Concentration of pNp (μmole/ml)	Concentration of added pA (μmole/ml)	Inhibitor (mmole/ml)	Hydrolysis (%)
0.09	-	-	70.0
0.36	-	-	44.6
0.90	-	-	9.5
0.09	0.067	-	71.0
0.09	3.3	-	11.4
0.09	-	0.23 (EDTA)	0
0.09	-	9.4 (cysteine)	0

* The digests were desalted on DEAE-cellulose without a preliminary fractionation of the digests into pN and pNp compounds. The pN and pNp compounds were resolved by 2-dimensional paper chromatography and the overall recoveries were usually 70 - 80 per cent. The lower recoveries obtained in these experiments (cf. Table XI) probably relate to the difficulties in obtaining suitable "blank" areas from the chromatograms.

tween the buffer and the 5'-nucleotides. The isolation and identification of these compounds has been described elsewhere (Lane, Diemer and Blashko, 1963) and quantitative data to show the extent of the formation of these compounds are presented in Table X.

(d) The stability of pNp and pN>p compounds in the phosphodiesterase hydrolyzates.

The stability of pNp compounds under the conditions of end group analysis with phosphodiesterase was investigated by incubating the compounds with snake venom phosphodiesterase at pH 9.2 and 37°. An aqueous solution of the pNp compounds was mixed with 2.5 ml of 1 M ammonium formate buffer, pH 9.2 and 2.5 ml of a solution of purified phosphodiesterase. The total volume of the digest was brought to 10 ml with distilled water and was incubated at 37° for 24 hours.

It was found that the pNp compounds were hydrolyzed to pN compounds and that the pN compounds were stable end products of the hydrolysis. The digests were fractionated into pN and pNp compounds by separation on DEAE-cellulose (Part II (ii)). The compounds were desalted on DEAE-cellulose (formate form) and were then resolved by one-dimensional paper chromatography in system A. The results of a typical experiment are shown in Table XI. The pNp compounds were completely stable when phosphodiesterase was omitted from the digests.

The effects of substrate concentration, product concentration and phosphodiesterase inhibitors, on the extent of hydrolysis of the pNp compounds was examined, and the results are shown in Table XII. These findings are con-

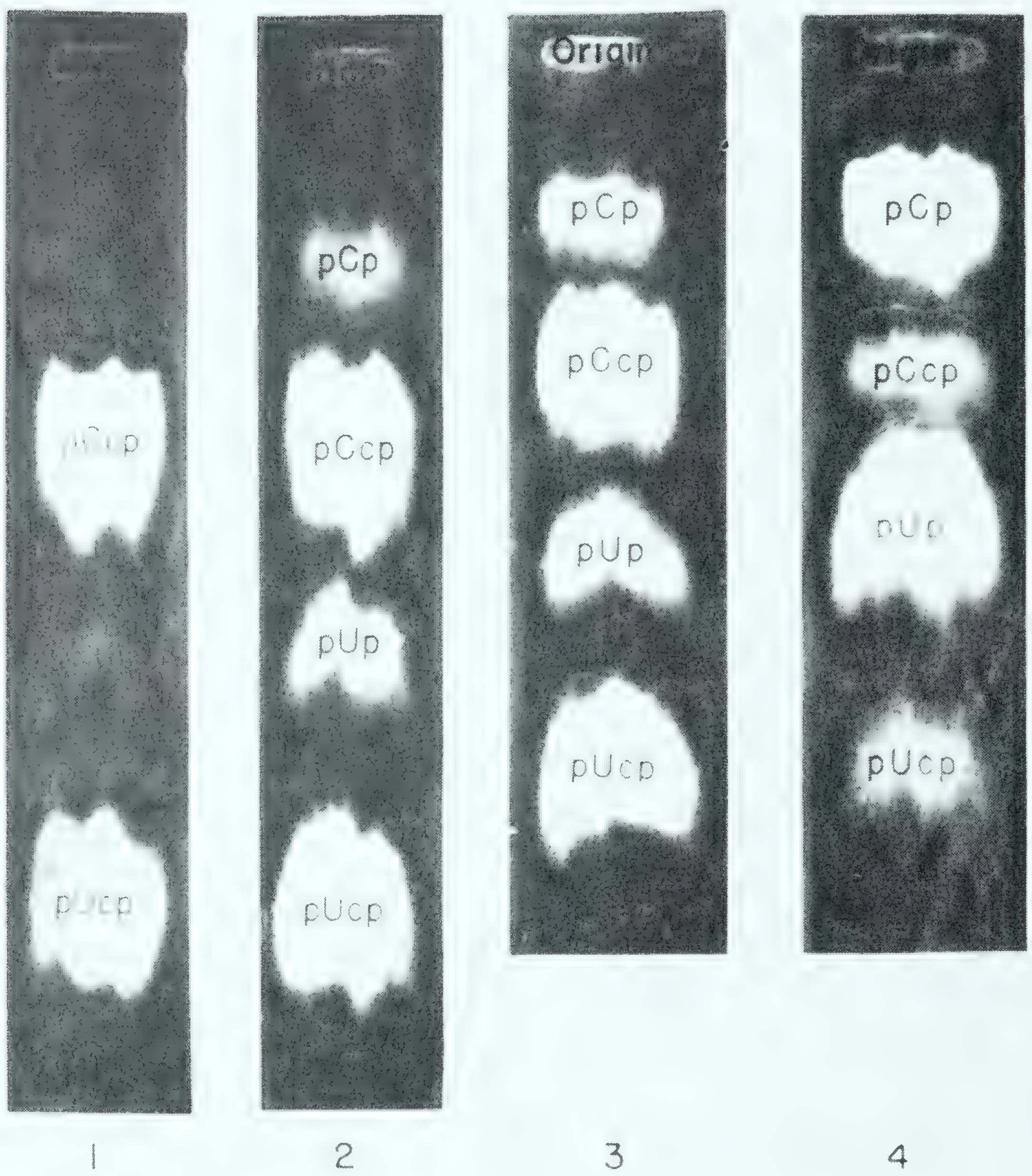
TABLE XIII

The spontaneous hydrolysis of pN>p compounds at pH 9.2 and 37°.

Time (hrs)	Yield of pN>p and pNp (s.u.)*				% hydrolysis	k (x10^{-3})
	pC>p	pCp	pU>p	pUp		
0	12.25	-			0	-
			16.80	-	0	-
48	11.91	1.33			10.0	2.3
			15.87	2.41	13.2	2.9
96	11.48	2.24			16.4	1.9
			15.00	3.80	20.2	2.3
144	11.12	2.68			19.3	1.5
			14.44	4.91	24.1	2.0
760	2.24	13.93			86.1	2.6
			1.80	16.50	90.2	3.0

* s.u. = O.D.(260 mμ) x volume.

Ultraviolet photographs of paper chromatograms showing the hydrolysis of pN>p compounds after various periods of time at pH 9.2 and 37°.



1 - 0 hrs.
2 - 48 hrs.
3 - 96 hrs.
4 - 760 hrs.

Figure 4.

sistent with the view that phosphodiesterase is itself responsible for the hydrolysis of pNp compounds. It hydrolyzes the 2' and 3' phosphomonoester bonds at about 1/2000th the rate at which it hydrolyzes diester bonds in ribonucleate chains. The failure to detect hydrolysis of Np compounds by phosphodiesterase is also consistent with the activating effect of a 5' phosphomonoester group in the hydrolysis of pNpN compounds by phosphodiesterase (pNpN compounds are hydrolyzed 20-fold faster than NpN compounds). A relevant situation has been noted with a 3'-phosphomonoesterase from rye-grass which appears to have a phosphodiesterase activity (Lane and Butler, 1959; Freeman, 1964). There was no detectable hydrolysis of pN>p compounds by venom phosphodiesterase under the same conditions which caused extensive hydrolysis of pNp compounds.

A time course study was undertaken in order to establish the rate of spontaneous conversion of pN>p compounds to pNp compounds under the conditions used for phosphodiesterase hydrolysis of ribonucleates. The reaction mixture contained 200 s.u. of a mixture of the pC>p and pU>p compounds in 0.5 ml of 0.25 M ammonium formate buffer, pH 9.2. Aliquots of 100λ were taken at 0, 48, 96, 144 and 760 hours after the beginning of the incubation and were chromatographed in system A. The ultraviolet photographs of several of these chromatograms are shown in Figure 4.

The chromatograms were eluted and the amounts of individual compounds were calculated as described elsewhere in the thesis. The results obtained are presented in Table XIII.

The rate constant for a presumed first order reaction

was determined algebraically for each "time", and these calculated values are also shown in Table XIII. The results indicate that pN>p compounds are subject to slow hydrolysis at pH 9.2. If the hydrolysis is presumed to be first order then the proportional hydrolysis of 2',3' cyclic phosphodiester bonds (dA/At=k) is about 0.002 mole per mole of phosphodiester bond per hour, and is about ten times faster than the mean rate of hydrolysis of the 3'-5' phosphodiester bonds measured for ribonucleates under the same conditions (i.e. phosphodiester bonds in the ribonucleates are hydrolyzed at a rate of about 0.02 mole per 100 moles per hour or 0.0002 mole per mole of phosphodiester linkage per hour).

A small degree of hydrolysis of pN>p compounds (less than 10%) to pNp compounds could be expected in the 24 hour period of an end group analysis but the concentration of the pN compounds in the digests would be expected to inhibit further hydrolysis of the pNp compounds to pN compounds. Since pN>p compounds are stable toward phosphodiesterase hydrolysis, there would be no reason to expect more than 10 per cent destruction of either pNp or pN>p compounds in the end group analyses by phosphodiesterase hydrolysis. It is not possible to account for the discrepancies of greater than 10 per cent which have been observed between nucleoside and diphosphonucleoside measurements in a number of analyses of fragmented ribonucleate preparations. They may be attributable to a removal of 2'- or 3'-phosphomonoester groups *before* release of the terminal residues from chain ends of ribonucleates. An enzyme of this type has recently been discovered by Richardson *et al.* (1964).

TABLE XIV

The end groups determined by phosphodiesterase hydrolysis of a badly degraded specimen of yeast ribonucleates from a commercial source.*

Constituent	mole/100 moles nucleotides	
Base:	Nucleosides	Diphosphonucleosides
a	1.74	0.68
g	1.47	0.22
c	0.84	0.42
u	1.08	0.95
Total	5.13	2.27

* The end group analysis was performed on 10 mg of ribonucleates in a total volume of 2.2 ml of 0.1 M ammonium chloride buffer. The digestion mixture contained 1 ml of phosphodiesterase which cleaved phosphodiester linkages at the rate of 7 µmoles per hour in a standard assay.

(ii) End group measurements.

(a) An extensively degraded ribonucleate specimen of commercial origin.

A preparation of badly degraded ribonucleates from yeast was purchased from Nutritional Biochemicals Co. and found to be polydisperse by sedimentation analysis. The sedimentation coefficient was about 2S in 0.1 M sodium chloride solution at a ribonucleate concentration of 0.3 per cent. The analysis for the end groups of type I structures in the preparation (Table VII) could not account for the small chain length expected from the sedimentation properties. The analyses for type II and III structures presented in Table XIV show that the bulk of the chains in preparations which have suffered extensive chemical and enzymic degradation are type II structures. The low yield of nucleoside diphosphates relative to nucleosides is attributable, in part, to some destruction of nucleoside diphosphates at the relatively high concentration of venom phosphodiesterase used in this analysis. It is also worth noting the possibility that such preparations could conceivably contain some endogenous nucleosides, although the quantity could not exceed 0.69 mole/100 moles as found in the analysis for type I structures (Table VII).

(b) A partially degraded specimen of E. coli high molecular weight ribonucleates.

A preparation of *E. coli* high molecular weight ribo-

TABLE XV

The distribution of bases in the end groups determined by snake venom phosphodiesterase hydrolysis of fragmented 18S + 28S ribonucleates from _E. coli_

Base	Nucleosides (mole/100 moles)		Diphosphonucleosides (mole/100 moles)**		
	1963*	1964***	1963*	1964***	
				cyclic	open
a	0.23	0.37	0.12	0.12	0.064
g	0.16	0.26	0.052	0.099	0.052
c	0.092	0.14	0.084	0.097	0.028
u	0.10	0.16	0.11	0.12	0.061
Total	0.58 ±0.02	0.93	0.37 ±0.02	0.64	

* The results of the analyses performed in 1963 have been described in detail elsewhere (Lane, Diemer and Blashko, 1963). The analyses in 1964 were performed on the same preparation of ribonucleates, after it had been stored for about one more year at room temperature.

** The diphosphonucleosides were desalted using charcoal in 1963 and using DEAE-cellulose in 1964.

*** The data presented here constitute the results of a single end group analysis using enzyme preparation 7 of Table XVI.

TABLE XVI

The end groups determined by snake venom phosphodiesterase hydrolysis of the 18S + 28S ribonucleates.

Nucleate prep.	Enzyme prep.	Enzyme activity*	mole/100 moles nucleotides										% Oligonucleotides
			A	G	C	U	Sum	pA>p	pG>p	pC>p	pU>p	Sum	
1	1	48	.061	.046	.028	.025	0.16	-	-	-	-	-	0
1	1	48	.053	.045	.028	.021	0.15	-	-	-	-	-	0
1	1	48(2x)	.062	.054	.031	.029	0.18	-	-	-	-	-	0
1	1	24	.045	.040	.024	.028	0.14	-	-	-	-	-	5
2	2	35	.054	.049	.033	.036	0.17	-	-	-	-	-	0
2	2	62	.045	.039	.024	.031	0.14	-	-	-	-	-	0
3	3	37	.062	.060	.033	.034	0.19	.020	.024	.039	.061	0.14	2
3	3	47	.058	.052	.029	.025	0.16	-	-	-	-	-	1.5
3	3	37(2x)	.081	.092	.045	.038	0.26	.042	.026	.079	.062	0.21	0.3
4	3	47	.052	.056	.033	.034	0.18	-	-	-	-	-	0.5
4	4	43	.072	.032	.015	.019	0.14	.017	.020	.031	.062	0.13	0
4	5	47	.035	.031	.016	.018	0.10	.022	.017	.036	.037	0.11	0
4	6	43	.035	.031	.019	.014	0.099	.026	.016	.037	.039	0.12	0
4	7	34	.038	.038	.015	.016	0.11	.021	.020	.043	.032	0.12	0
4	8	35	.086	.071	.033	.052	0.24	.037	.033	.056	.069	0.20	1.1
5	7	34	.059	.061	.027	.032	0.18	.039	.029	.077	.056	0.20	0.3
Mean values and standard deviation			.056 ± .014	.050 ± .016	.027 ± .008	.028 ± .009	0.16 ± 0.043	.028 ± .009	.023 ± .006	.050 ± .018	.052 ± .013	0.15 ± 0.039	

* μmole phosphodiester linkages hydrolyzed in the first hour of an end group analysis at pH 9.2. Incubation was extended to 48 hours with an addition of fresh enzyme at 24 hrs. in those experiments designated (2x),(see footnote to Table IX).

nucleates which had been stored for about three years as a powder at room temperature was examined by sedimentation analysis and shown to have the properties characteristic of "subunit" ribonucleates. The preparation was polydisperse and had a mean $S^{o}_{20,w}$ of 8 when sedimented at an ionic strength of 0.04. The preparation was extensively examined by end group methods in 1963 and it was found that the degradation apparent from sedimentation analysis was also evident from measurements of the amount of end groups found for type II and III structures. This work has been discussed extensively elsewhere (Lane _et_ _al_., 1963). The preparation was examined about one year later, in 1964, after further storage in the powder state at room temperature. The data from these analyses are shown in Table XV and it is evident that the relative rate of increase of individual end groups over a four year period was quite uniform. This suggests that fragmentation occurs spontaneously during storage in the powder state with the appearance of new type II and III structures. As mentioned earlier in the thesis, there is no evidence for the production of type I structures during fragmentation of ribonucleate chains.

(c) Undegraded 18S + 28S ribonucleates from wheat germ.

The end group analyses of five different preparations of 18S + 28S ribonucleates from wheat germ are shown in Table XVI. The data were obtained in 16 independent experiments, and indicate that the amounts of end groups

TABLE XVII

The end groups produced by spontaneous hydrolysis of ribonucleates during end group analyses with phosphodiesterase calculated from the time-course of hydrolysis and the observed mean rate of spontaneous hydrolysis.

Time (hrs)	Rate of hydrolysis by phosphodiesterase: 35 μmole phosphodiester linkages per hour		Rate of hydrolysis by phosphodiesterase: 23 μmole phosphodiester linkages per hour	
	% hydrolysis by phosphodiesterase**	End groups from spont. hydrolysis***	% hydrolysis by phosphodiesterase**	End groups from spont. hydrolysis***
1	31	85x0.023*= 0.020	20	90x0.023*= 0.021
2	46	61x0.023*= 0.014	32	74x0.023*= 0.017
3	58	48x0.023*= 0.011	41	63x0.023*= 0.014
4	66	38x0.023*= 0.0087	48	55x0.023*= 0.013
5	72	31x0.023*= 0.0071	55	48x0.023*= 0.011
6	78	25x0.023*= 0.0058	62	41x0.023*= 0.0094
7	84	19x0.023*= 0.0043	68	35x0.023*= 0.0080
24	100	(8x17x0.023*= 0.031)	95	18x17x0.023*= 0.070
Total end groups***		0.071 (0.102)		0.16

* Mean rate of spontaneous hydrolysis at pH 9.2 and 37° is about 0.023 mole phosphodiester linkages per 100 moles total linkages per hour.

** The extent of hydrolysis was measured by the liberation of TCA-soluble material in the standard assay extended to the times indicated. The extent of hydrolysis after 24 hours is based on the oligonucleotide residue found in the end group analyses.

*** Expressed as mole/100 moles nucleotides.

TABLE XVIII

The effect of incomplete hydrolysis by phosphodiesterase on the end group measurements.

End group:	moles/100 moles nucleotides	
	Complete hydrolysis*	Incomplete hydrolysis**
A	0.051 ± 0.012	0.063 ± 0.014
G	0.041 ± 0.008	0.062 ± 0.015
C	0.023 ± 0.007	0.032 ± 0.006
U	0.023 ± 0.007	0.035 ± 0.008
Total nucleosides	0.14 ± 0.028	0.19 ± 0.039
pA>p	0.022 ± 0.003	0.034 ± 0.009
pG>p	0.018 ± 0.002	0.028 ± 0.003
pC>p	0.037 ± 0.004	0.063 ± 0.017
pU>p	0.042 ± 0.012	0.062 ± 0.005
Total diphosphonucleosides	0.12 ± 0.007	0.19 ± 0.028

* Summary of 9 analyses for nucleosides and 4 analyses for diphosphonucleosides.

** Summary of 7 analyses for nucleosides and 4 analyses for diphosphonucleosides.

varied between 0.1 and 0.2 mole per cent with different ribonucleate preparations. The diphosphonucleosides were found to be exclusively pN>p rather than pNp compounds.

The data on undegraded ribonucleates are believed to reflect the fact that some spontaneous hydroxyl-ion catalyzed cleavage is unavoidable at the pH value of 9.2 needed for venom phosphodiesterase hydrolysis and that all the nucleosides and diphosphonucleosides derive from nascent chain ends formed in the course of the end group analysis itself. The spontaneous hydrolysis rate at pH 9.2 and 37° has been measured (Part V(i)) and could be expected to contribute at least 0.07 mole per 100 moles of total nucleotides to the end group measurements on the basis of the mean rate of hydrolysis over a 100 hour period at pH 9.2 and 37°. This is illustrated by the data of Table XVII where it can be seen that the amount of spontaneous hydrolysis during an analysis can be calculated from a knowledge of the time course of hydrolysis and a mean value for the rate of spontaneous hydrolysis. It is notable that the calculated contribution of spontaneous hydrolysis is greater for a slower hydrolysis rate by phosphodiesterase, and that this was observed in practice, since higher values for end groups were obtained when hydrolysis by phosphodiesterase was incomplete (Table XVIII). This was also apparent from the decreased quantity of end groups observed when the activity of the phosphodiesterase was increased (cf. the analysis of nucleate preparation 2 at two different concentrations of

phosphodiesterase preparation 2, Table XVI).

The variation between 0.1 and 0.2 mole of end group per 100 moles of nucleotides for different ribonucleate preparations can perhaps be partly attributed to the presence in the ribonucleates of variable small amounts of metal ions such as magnesium which has been shown to drastically alter the rate of spontaneous hydrolysis at pH 9.2 and 37° (see Part V(ii)). The calculated contribution of spontaneous hydrolysis tothe end group values is not sufficient to fully account for all the end groups measured in the analyses but this is not surprising since the rate of spontaneous hydrolysis might be expected to diminish with chain length and could, in the early stages, be greater than the mean value used in the calculation of end groups resulting from spontaneous hydrolysis. Additionally, very small amounts of ions like magnesium would be expected to make their contribution to an accelerated spontaneous hydrolysis rate early in the analysis and make only a small contribution to the mean rate over a long period.

It is noteworthy that about 70 per cent of the nucleosides are purine nucleosides and about 70 per cent of the diphosphonucleosides are pyrimidine diphosphonucleosides in the analyses of Table XVI. This is in precise agreement with what would be expected if the end groups had resulted from spontaneous hydrolysis at pH 9.2 and 37° (see Part V (i)).

The values in the neighbourhood of 0.1 mole per 100 moles of nucleotides can be accounted for reasonably well

TABLE XIX

Nucleoside end groups determined by snake venom phosphodiesterase hydrolysis of s-ribonucleates from wheat germ.*

Substrate concentration (%)	Enzyme activity**	mole/100 moles nucleotides***				
		A	G	C	U	Total
0.5	54	0.042	0.060	0.039	0.029	0.17

* The analysis for diphosphonucleosides was not possible because of contamination of the s-ribonucleate preparation with phenolic compounds. It was nonetheless evident that the quantity of diphosphonucleosides and unhydrolyzed oligonucleotides could not have exceeded 1% of the total nucleotide material in the hydrolyzate.

** Activity expressed as µmole phosphodiester linkages cleaved in the first hour of the end group analysis.

*** The nucleoside fraction also contained about 0.20 mole of 1-methyl-pA per 100 moles of nucleotides. The mobility of 1-methyl-pA in system A is about the same as that of pC.

as arising from spontaneous hydrolysis. The value of about 0.2 mole per 100 moles would seem to be accounted for in this way if it is assumed that there is an accelerated rate of spontaneous hydrolysis due to the presence of small amounts of counterions such as magnesium in some ribonucleate preparations (see Part V(ii)). Alternatively, there might be a retarded rate of phosphodiesterase hydrolysis in the later stages of hydrolysis in some instances due to small amounts of inhibitory materials in some phosphodiesterase or ribonucleate preparations. This latter possibility is supported by the observation that some resistant oligonucleotide residues were only slowly hydrolyzable even after reincubation with a large excess of fresh enzyme. A comparison of the analysis of nucleate preparations 4 and 5 using the same amount of phosphodiesterase preparation 7 serves to illustrate these contentions, (see Table XVI).

(d) Undegraded s-ribonucleates from wheat germ.

Wheat germ s-ribonucleates, isolated and purified according to the method of Glitz and Dekker (1963), were analyzed for end groups by phosphodiesterase hydrolysis and the results are presented in Table XIX. The quantity of end groups found for type II and III structures after phosphodiesterase digestion of the s-ribonucleates is probably ascribable to spontaneous hydrolysis in the course of the analysis itself. This deduction is supported by the fact that the distribution of the bases in the nucleoside

fraction obtained after hydrolysis is the same as would be expected from spontaneous hydrolysis at pH 9.2. It may be concluded that the s-ribonucleates are exclusively type I structures.

TABLE XX

The end groups produced by spontaneous hydrolysis of
18S + 28S ribonucleates at pH 9.2 and 37^{o}.*

RNA prep.	Time (hrs)	[Buffer]**	[SC]**	mole/100 moles nucleotides									
				A	G	C	U	Total N	pA>p	pG>p	pC>p	pU>p	Total pN>p
1	0			.10	.063	.035	.034	0.23	-	-	-	-	-
1	27	0.28 M AC	-	.38	.32	.15	.18	1.0	-	-	-	-	-
1	96	"	-	1.1	.87	.40	.47	2.8	.46	.27	.52	.76	2.0****
2	0			.054	.049	.033	.036	0.17	-	-	-	-	-
2	5	0.28 M AC	-	.088	.082	.046	.049	0.26	-	-	-	-	-
2	98	"	-	.93	.71	.26	.39	2.3	.41	.12	.41	.51	1.4****
2***	0			.13	.12	.071	.082	0.40	-	-	-	-	-
2***	5	0.28 M AC	-	.32	.27	.14	.21	0.94	.21	.067	.18	.24	0.70****
3	0			.035	.031	.018	.014	0.098	.024	.015	.035	.038	0.11
3	100	0.25 M AF	-	.61	.71	.31	.31	1.9	.32	.26	.37	.59	1.6
3	100	.025M AF	-	.19	.14	.10	.066	0.50	.033	.049	.10	.091	0.27
3	100	"	.225 M	.14	.18	.065	.072	0.46	.10	.093	.071	.16	0.42

---

* Ribonucleate concentration was 0.5 - 0.7%.

** AC (=ammonium chloride), AF (=ammonium formate), SC (=sodium chloride).

*** Converted to the Mg^{++}-counterion form by precipitating preparation 2 from 1 M magnesium chloride solution.

**** Desalted on charcoal.

PART V - THE CLEAVAGE OF 18S + 28S RIBONUCLEATES UNDER A VARIETY OF CONDITIONS COMMONLY EMPLOYED IN STUDIES OF RIBONUCLEATES.

The rates of spontaneous hydrolysis of ribonucleates were determined by preincubating the ribonucleates for a suitable period of time under selected conditions before adjusting the pH to 9.2 and hydrolyzing with venom phosphodiesterase. The quantity of end groups observed by hydrolysis with phosphodiesterase, without preincubation, was subtracted from the quantity measured after specific conditions of preincubation to arrive at the quantity of new end groups formed as the result of the preincubation. Many of these experiments were performed prior to the development of adequate methods for recovery of diphosphonucleosides, and for this reason, the measurements of nucleosides are considered more reliable than measurements of diphosphonucleosides.

(i) Spontaneous hydrolysis at pH 9.2 and 37°.

The data of Table XX show that there is some difference in the rate of spontaneous hydrolysis among various ribonucleate preparations but that the differences are not great. It is also notable that the concentration of ammonium formate buffer had a marked influence on the rate of spontaneous hydrolysis since the rate of hydrolysis in 0.25 M ammonium formate, pH 9.2, was about four times greater than the rate in 0.025 M ammonium formate buffer, pH 9.2.

This influence of buffer concentration was not simply an ionic strength effect since replacement of ammonium formate by sodium chloride (i.e. 0.025 M ammonium formate - 0.22 M sodium chloride) gave a hydrolysis rate in accordance with the decreased ammonium formate concentration. This probably reflects the contribution of ammonia and/or ammonium formate to the hydrolytic catalysis.

The influence of buffer concentration on the rate of spontaneous hydrolysis suggests that the quantities of end groups observed in the analyses of Table XVI might be substantially reduced by conducting phosphodiesterase hydrolysis in 0.15 M sodium chloride solution and titrating liberated acid groups by addition of a strong alkali.

(ii) The labilizing effect of magnesium counterion.

A typical preparation of 18S + 28S ribonucleates was assayed for end groups of type II and III structures and its rate of spontaneous hydrolysis at pH 9.2 and 37° was determined. A sample of the same preparation was then converted to the magnesium counterion form and again analyzed for end groups and spontaneous hydrolysis at pH 9.2 and 37°. The data obtained in the end group analyses are presented in Table XX.

From these data it can be concluded that the rate of spontaneous hydrolysis is about 5-fold greater when ribonucleates are in the magnesium counterion form rather than

TABLE XXI

The end groups produced by spontaneous hydrolysis of 18S + 28S ribonucleates at different pH values and temperatures.

pH	Temp.	Time	[Buffer]	[SC]	mole/100 moles nucleotides									
					A	G	C	U	Total N	pA>p	pG>p	pC>p	pU>p	Total pN>p
		0			.10	.064	.035	.034	0.23	-	-	-	-	-
6.8	100	1	.05 M PP	-	.12	.13	.044	.060	0.35	-	-	-	-	-
6.8	100	5	"	-	.22	.34	.15	.11	0.82	.22	.16	.18	.18	0.74
		0			.035	.031	.019	.014	0.099	.036	.016	.037	.039	0.12
5.2	37	100	.02 M SA	.22 M	.075	.067	.020	.031	0.19	.069	.047	.048	.082	0.25
		0			.072	.032	.015	.019	0.14	.017	.020	.031	.062	0.13
4	37	100	-	.15 M	.11	.069	.024	.039	0.24	.022	.034	.031	.058	0.14
		0			.035	.031	.019	.014	0.099	.036	.016	.037	.039	0.12
2*	30	5	-	-	.28	.39	.29	.21	1.2	.29	.24	.18	.19	0.90
1**	30	5	-	-	1.2	1.8	1.6	1.2	5.8	1.2	1.8	1.3	1.2	5.5
13***	30	5	-	-	3.6	5.0	3.2	2.2	14.0	2.1	2.2	2.1	2.1	8.3

* 70% pNp, 30% pN>p.

** 100% pNp.

*** 100% pNp, and 80% of the oligo-residue was eluted from DEAE-cellulose with the eluent used for elution of nucleoside diphosphates (0.35 M TRIS formate in 7 M urea). There was an oligonucleotide residue only in the experiment performed at pH 13. The residue amounted to 15% of the total hydrolysis products.

TABLE XXII

Rates of hydrolysis of 18S + 28S ribonucleates at various pH values and temperatures, based on measurements of nucleoside end groups after phosphodiesterase hydrolysis.

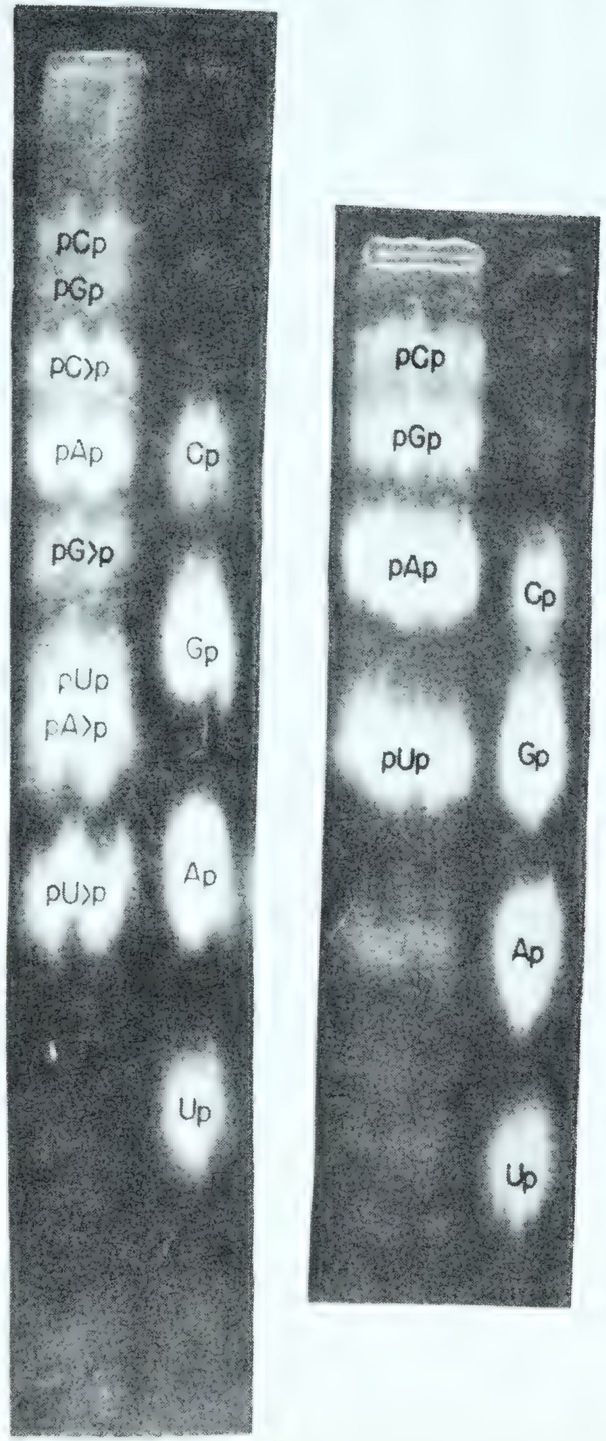
pH	Temp.	Time (hrs)	[Anion] **	mole% phosphodiester bonds cleaved per hour	Number of scissions per nucleate chain per hour
1.0	30	5	0.15 M HC-SC(2:1)	1.1	15
2.0	30	5	0.16 M SC-HC(15;1)	0.24	3.1
4.0	37	100	0.15 M SC	0.0010	0.013
5.2	37	100	0.22 M SA-SC(1:10)	0.00090	0.012
6.8	100	1	0.05 M PP	0.12	1.6
6.8	100	5	0.05 M PP	0.12	1.6
9.2	37	5	0.25 M AC	0.018	0.23
9.2	37	98	0.25 M AC	0.022	0.29
9.2	37	27	0.25 M AF	0.028	0.36
9.2	37	96	0.25 M AF	0.029	0.38
9.2*	37	5	0.25 M AF	0.11	1.4
9.2	37	100	0.25 M AF	0.018	0.23
9.2	37	100	0.025 M AF	0.0040	0.052
9.2	37	100	0.25 M AF-SC(1:10)	0.0040	0.047
13.0	30	5	0.15 M SC-SH(1:2)	2.8	36

* The ribonucleates were in the Mg^{++}-counterion form.

** HC - hydrochloric acid; SC - sodium chloride; SA - sodium acetate; PP - potassium phosphate; AC - ammonium chloride; AF - ammonium formate; SH - sodium hydroxide.

Figure 5.

Ultraviolet photographs illustrating the one-dimensional paper chromatographic resolution of pN>p and pNp compounds.



in the sodium counterion form.

(iii) The effect of heating at 100° and neutral pH.

A sample of 18S + 28S ribonucleates, which had previously been assayed for end groups by venom phosphodiesterase hydrolysis, was incubated at pH 6.8 and 100°. The preparation consisted of two components with $S_{20,w}$ values of 13 and 16.5 (0.05 M phosphate buffer, 0.5% ribonucleate concentration) before heating, and was polydisperse having a mean $S_{20,w} = 9.6$ after heating for one hour. New end groups of type II and III structures appeared at a mean rate of 0.12 mole per 100 moles of nucleotides per hour, (see Tables XXI, XXII). This shows that the "subunit" structures formed by heating ribonucleates at neutral pH (Hall and Doty, 1959) result from the cleavage of phosphodiester bonds rather than from a disruption of secondary forces in an aggregate structure.

Typical ultraviolet photographs illustrating the one-dimensional paper chromatographic resolution of pN>p and pNp compounds derived from these heating experiments, are shown in Figure 5.

(iv) The hydrolysis rate at different pH values and temperatures.

Ribonucleate preparations were incubated at various pH values and temperatures commonly used in the studies of ribonucleates and their derivatives. It is apparent that the hydrolysis rate in 0.1 M hydrochloric acid is

more than one-third the rate in 0.1 M sodium hydroxide solution at 30° (see Table XXII). This is not generally recognized since incubation in 0.1 M hydrochloric acid is commonly used as a means of cleaving 2',3' cyclic phosphate ends of oligonucleotides on the assumption that 3'-5' phosphodiester linkages are not cleaved under these conditions whereas 0.1 M sodium hydroxide is employed to achieve complete hydrolysis of ribonucleates. It is evident that these two treatments are not as dissimilar as generally presumed. It is also apparent that the pH of maximum stability is probably on the acid side of neutrality at 37° since the hydrolysis rate at pH 4 is 20-fold smaller than the rate at pH 9.2 (Table XX). This agrees with indirect measurements recently made by Ginoza *et al.* (1964) on the hydrolysis of tobacco mosaic virus ribonucleate.

TABLE XXIII

End group and sedimentation analyses of the 18S + 28S ribonucleates stored in the powder state.

Time (days)	Temp. °C	$S_{20,w}$ slow fast	mole/100 moles nucleotides									
			A	G	C	U	Total N	pA>p	pG>p	pC>p	pU>p	Total pN>p
0	-20	15 + 20 didisperse	.035	.031	.016	.018	0.10	.022	.017	.036	.037	0.11
81	-20	15 + 20 didisperse	.035	.031	.019	.014	0.099	.026	.016	.037	.039	0.12
91*	-20	15 + 20 didisperse	.038	.038	.015	.016	0.11	.021	.020	.043	.032	0.12
88*	+20	14 polydisperse	.067	.089	.046	.041	0.24	.043	.034	.072	.066	0.22

* The same enzyme preparation was used for the end group analyses in these experiments, and the paper chromatograms and spectral analyses from these two experiments have been presented in Figure 3 as A(-20°) and B(+20°).

TABLE XXIV

Sedimentation analyses of 18S + 28S ribonucleates after storage in the powder state at -20°.

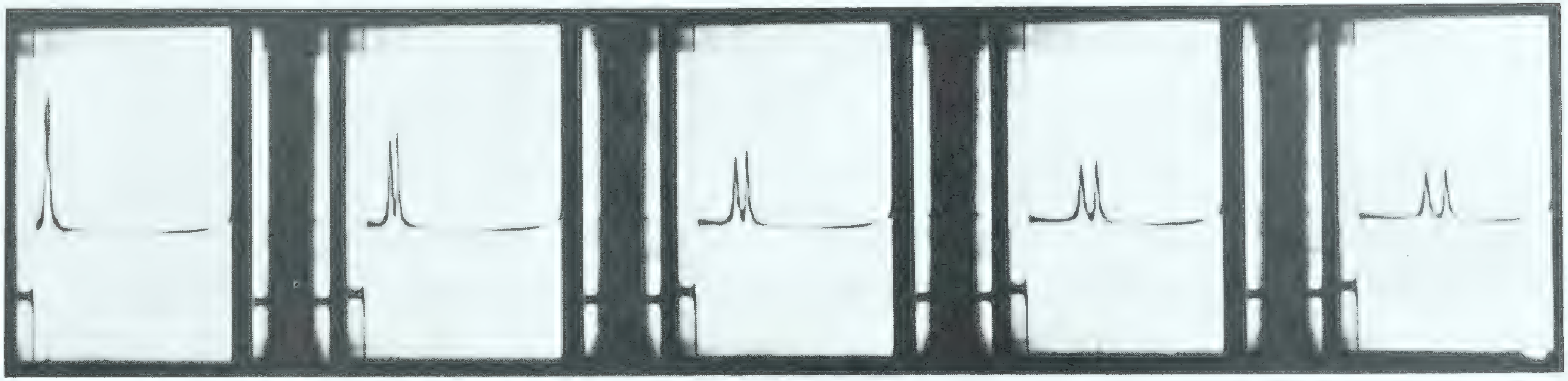
Days stored	Slow component	Fast component
0*	50% - 15.7S ($S_{20,w}$ = 15.8)	50% - 19.2S ($S_{20,w}$ = 19.3)
153*	57% - 16.5S ($S_{20,w}$ = 15.0)	43% - 20.8S ($S_{20,w}$ = 19.0)
153**	66% - 13.6S ($S_{20,w}$ = 13.0)	34% - 16.5S ($S_{20,w}$ = 15.7)

* Sedimentation was conducted in 0.15 M sodium chloride solution with a ribonucleate concentration of 0.25% and was observed by the schlieren optical system.

** Sedimentation was conducted in 0.15 M sodium chloride solution with a ribonucleate concentration of 0.50% and was observed by the schlieren optical system.

Schlieren photographs from sedimentation studies.

(a) Preparation of 18S + 28S ribonucleates from wheat germ stored 153 days at -20°.



(b) Preparation of 18S + 28S ribonucleates from wheat germ stored 88 days at +20°.

PART VI - THE STORAGE OF 18S + 28S RIBONUCLEATES IN THE POWDER STATE.

During the course of the investigations reported in the thesis there were indications that the high molecular weight ribonucleates underwent spontaneous degradation when stored as a powder at room temperature (see Table XV). In order to verify the occurrence of such a breakdown, a preparation of 18S + 28S ribonucleates was analyzed for end groups immediately after isolation, and again at various times after being stored either at room temperature or at -20°. The end group data obtained from these analyses are shown in Table XXIII. Sedimentation analyses were also performed in parallel with end group analyses, and a summary of these data also appears in Table XXIII.

The sedimentation data presented in Table XXIV support the end group data of Table XXIII since there appears to be only very slight degradation at -20° judging by the constancy of the areas corresponding to the 18S + 28S components in schlieren photographs. It is noteworthy that the relative amounts of material in the slow and fast peaks observed with schlieren optics is critically dependent on ribonucleate concentration.

The photographs of Figure 6 show the sedimentation patterns of the same ribonucleate preparation when it was stored (a) at -20°, and (b) at +20°. These photographs clearly support the end group data of Table XXIII since there is evidence of extensive degradation after storage at +20° for 88 days, but no evidence of degradation dur-

ing storage at -20°.

It was concluded from these results that no significant amount of degradation occurred if the nucleates were stored as a powder at -20° but that considerable degradation occurred if the nucleates were stored in the powder state at room temperature. The end group data demonstrate that the disintegration at room temperature reflects cleavage of phosphodiester bonds in ribonucleate chains, and not merely a dissociation of "subunit" chains held together in an aggregate structure by secondary forces.

PART VII - SUMMARY

1. High molecular weight ribonucleates were isolated from wheat germ by a modification of the phenol method of Colter and Brown (1956). The procedure was suitable for preparing 5 grams of pure 18S + 28S ribonucleates in one day.

2. Alkali hydrolysis of the 18S + 28S ribonucleates yielded nucleosides and nucleoside diphosphates in quantities indicative of a mean chain length of 1300 nucleotide residues for chains having a type I (pNpN----pNpN-----pNpN) structure. The recovery of nucleosides and nucleoside diphosphates of each of adenine, guanine, cytosine and uracil has established that there are at least four distinct ribonucleate chains in the preparations.

3. Phosphodiesterase of Russell Viper Venom was partially purified by the method of Koerner and Sinsheimer (1957). Further purification was achieved by using a modification of the original procedure of Hurst and Butler (1951). The recovery of phosphodiesterase activity from whole venom was about 15 per cent. The overall purification of phosphodiesterase was only 4-fold, but the resulting enzyme was free of phosphomonoesterase and endonuclease activities.

4. Phosphodiesterase hydrolysis of the 18S + 28S ribonucleates yielded nucleosides and nucleoside diphosphates

which derived from the chain ends of type III structures (i.e. NpNp----pNpN----pN>p) but evidence was presented to show that the type III structures were not present in the preparations prior to incubation at the slightly alkaline pH value of 9.2 needed for phosphodiesterase hydrolysis.

5. Phosphodiesterase hydrolysis of 18S + 28S ribonucleates following preincubation under conditions which induce cleavage of phosphodiester bonds was used to measure the rate of hydrolysis of the ribonucleates at several different pH values and temperatures. Ribonucleate cleavage was characterized by the formation of new type III (NpNp----NpNp----NpN>p) and type II (NpNp---NpNp---Np) structures. The type III structures predominated under mild hydrolytic conditions and type II structures predominated after more drastic treatment. The pH value of maximum stability appeared to be on the acid side of neutrality.

6. Phosphodiesterase hydrolysis has incidentally shown that at least 99.5 per cent of the constituent nucleosides of the 18S + 28S ribonucleates bear 5'-phosphate substituents.

7. Sedimentation analysis and end group analysis for type II and III structures were employed to establish that the

18S + 28S ribonucleates could be stored for three months at -20° in the powder state without substantial degradation. Storage in the powder state at +20° was observed to result in considerable degradation over a period of three months and the degradation was shown to be attributable to a cleavage of phosphodiester bonds.

BIBLIOGRAPHY

Allen, F.W. (1962), "Ribonucleoproteins and Ribonucleic Acids", Elsevier, Amsterdam.

Aronson, A.I., and McCarthy, B.J. (1961), Biophys. J. 1, 215.

Brown, D.M., and Todd, A.R. (1952a), J. Chem. Society, 44.

Brown, D.M., and Todd, A.R. (1952b), J. Chem. Society, 52.

Brown, D.M., and Todd, A.R. (1954), J. Chem. Society, 2040.

Brown, R.A., Ellem, K.A.O., and Colter, J.S. (1960), Nature 187, 509.

Cantoni, G.L., Ishikura, H., Richards, H.H., and Tanaka, K. (1963), Cold Spr. Harb. Symp. on Quant. Biol. XXVIII, 123.

Chao, F.C., (1961), Biochim. Biophys. Acta 53, 64.

Cohn, W.E., and Volkin, E. (1952), Arch. Biochem. Biophys. 35, 465.

Colter, J.S., and Brown, R.A. (1956), Science 124, 1077.

Crestfield, A.M., and Allen, F.W. (1956), J. Biol. Chem. 219, 103.

Davis, F.F. (1962), Biochim. Biophys. Acta 61, 138.

Dekker, C.A., and Schachman, H.K. (1954), Proc. Nat. Acad. Sci. U.S. 40, 894.

Doty, P., and Rice, S.A. (1955), Biochim. Biophys. Acta 16, 446.

Dunn, D.B.,(1959), Biochim. Biophys. Acta 34, 286.

Fraenkel-Conrat, H., and Singer, B. (1962), Biochemistry (A.C.S.) 1, 120.

Freeman, K.B. (1964), Can. J. Biochem. 42, 1099.

Ginoza, W., Hoeller, C.J., Vessey, K.B., and Carmack, C. (1964), Nature 203, 606.

Glitz, D.G., and Dekker, C.A. (1963), Biochemistry (A.C.S.) 2, 1185.

Goldstein, J., Bennett, T.P., and Craig, L.C. (1964), Proc. Nat. Acad. Sci. U.S. 51, 119.

Hall, B., and Doty, P. (1959), J. Mol. Biol. 1, 111.

Holley, R.W. (1963), Bioch. Biophys. Res. Comm. 10, 186.

Holley, R.W., Madison, J.T., and Zamir, A. (1964), Bioch. Biophys. Res. Comm. 17, 389.

Hurst, R.O., and Butler, G.C. (1951), J. Biol. Chem. 193, 91.

Koerner, J.F., and Sinsheimer, R.L. (1957), J. Biol. Chem. 228, 1049.

Kornberg, A. (1957), Adv. in Enzymology 18, 191.

Lane, B.G., and Allen, F.W. (1961), Biochim. Biophys. Acta 47, 36.

Lane, B.G. (1963), Biochim. Biophys. Acta 72, 110.

Lane, B.G., Diemer, J., and Blashko, C.A. (1963), Can. J. Bioch. Physiol. 41, 1927.

Markham, R., and Smith, J.D. (1952a), Biochem. J. 52, 552.

Markham, R., and Smith, J.D. (1952b), Biochem. J. 52, 558.

Markham, R., and Smith, J.D. (1952), Biochem. J. 52, 565.

Otaka, E., Oota, Y., and Osawa, S. (1961), Nature 191, 598.

Reich, E., Franklin, R.M., Shatkin, A.J., and Tatum, E.L. (1962), Proc. Nat. Acad. Sci. U.S. 48, 1238.

Richardson, C.C., Inman, R.B., and Kornberg, A. (1964), J. Mol. Biol. 9, 46.

Spirin, A.S. (1960), J. Mol. Biol. 2, 436.

Takanami, M., (1958), Biochim. Biophys. Acta 29, 430.

Taylor, J.H. (1963), "Molecular Genetics" part I, 65. Academic Press, New York.

Tomlinson, R.V., and Tener, G.M. (1963), Biochemistry (A.C.S.) 2, 697.

Welsh, R.S. (1962), Proc. Nat. Acad. Sci. U.S. 48, 887.

Zubay, G. (1962), J. Mol. Biol 4, 347.

APPENDIX

Chemical preparations.

1. Ammonium formate buffer, 1 M, pH 9.2.

Concentrated ammonia (133.3 ml) was mixed with 500 ml of water and 42.2 ml of concentrated formic acid solution. The volume of the ammonium formate solution was brought to 950 ml by addition of water and the pH was adjusted to 9.2 by the dropwise addition of concentrated formic acid. The final volume was then adjusted to 1000 ml with water.

2. Phosphate buffer, 0.05 M, pH 6.8.

8.7 gm K_2HPO_4 and 6.8 gm KH_2PO_4 were dissolved in water to give a final solution volume of 2 litres.

3. Water-saturated phenol solution.

630 gm of phenol crystals were melted at 50° and then mixed with 70 ml of water.

4. Ammonium acetate buffer, 0.5 M, pH 4.

Concentrated acetic acid (60 ml) was mixed with 540 ml of water and 33.4 ml of concentrated ammonia solution. The pH value was adjusted to 4 by the dropwise addition of concentrated acetic acid and the final volume was brought to 1000 ml with water.

5. Pyridinium formate, 1 M, pH 4.5.

79 ml pyridine and 42 ml concentrated formic acid were added to 800 ml water and the volume of the solution was brought to 1000 ml with water.

6. TRIS formate buffer, 1 M, pH 7.8.

Concentrated formic acid (60 ml) was added to 1500 ml of water containing 242 gm TRIS, and the final volume was brought to 2000 ml with water.

B29828